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Investigation of In-Vivo Effect of St. John's Wort (Hypericum Perforatum) In Lung Cancer

Aktaş S*, Erol A, Serinan EO, Gökbayrak OE and Altun Z

Department of Basic Oncology, Institute of Oncology, Dokuz Eylul University, Izmir, Turkey

***Address for correspondence:**
Aktaş S, Department of Basic Oncology, Institute of Oncology, Dokuz Eylul University, Izmir, Turkey; Email: safiyeaktas@gmail.com

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Abstract

Due to limitations in the treatment of lung cancer, finding natural compounds from plants can provide an alternative treatment for lung cancer. St. John's Wort (SJW) has anti-proliferative and pro-apoptotic properties that can be used in lung cancer treatment. The aim of this study is to explore antitumor effect of SJW in lung cancer in vivo animal model. 35 animals; 7 animals in each group were randomized as control, Doxorubicin, SJW early treatment, SJW treatment, and doxorubicin+SJW groups. After 7 days sacrification was performed. Tumor diameter did not show statistically significant change but in all four-group compared with control group; tumor tissue showed prominent necrosis and apoptosis. No histologic changes observed in other tissues. Biochemistry did not show organ insufficiency.

SJW is shown to have antitumoral effect in subcutaneous xenograft lung cancer in vivo model in nude mice. Dose was obtained comparing with DOX. In combination with DOX, there were no synergistic increase in anti-tumo effect. SJW might be a candidate antineoplastic supplementation in lung cancer.

Introduction

Lung cancer is one of the most common malignant tumors in the world, consisting of pathologically and clinically diverse subtypes [1-4]. Due to limitations in the treatment of lung cancer, finding natural compounds from plants can provide an alternative treatment for lung cancer [5, 6]. St. John's Wort (St. John's Wort = SJW = Hypericum Perforatum) is also one of these plants used. SJW has previously taken its place in the literature as a herbaceous herb used in the treatment of fibrosis, neuralgia, depression and anxiety as an alternative to classical antidepressants [7]. SJW's most well-known bioactive compounds are hypericin, hyperoside and hyperforin. Hypericin; SJW started to take part in cancer studies after it was revealed that reactive oxygen species (ROS) are produced in cells and photodynamic therapy (PDT) that induces apoptosis, necrosis or autophagy is a photo-sensitizer that can be used [7,8]. After the use of SJW in cancer research, it has also been revealed that the accumulation of hypericin in neoplastic tissue is significantly higher than normal tissue and can be used as an effective fluorescent marker for tumor detection and imaging in photodynamic diagnosis (PDD) [8]. However, drug interaction of hypericin and hyperforin, which are active components of SJW, with anticancer drugs have been reported in various studies. SJW modulates the expression of multidrug resistance-1 (MDR-1), which is the main multidrug resistance mechanism responsible for the failure of chemotherapy [8].

SJW has anti-proliferative and pro-apoptotic properties that can be used in lung cancer treatment [7,8]. The proliferation inhibitory effect and apoptosis-inducing effect of hyperoside in lung cancer have been shown in various studies [9,10]. Although there are studies in the literature showing the anti-proliferative and pro-apoptotic effects of SJW in many different types of cancer, there is no study evaluating the combination of SJW with an agent used in traditional lung cancer treatment.

In the in-vitro part of the study, the 5µg/ml-50mg/ml dose range of SJW on the LLC lung cancer cell line was applied at 24, 48, 72 hours incubation times and WST-1 cell viability test was performed. The LD50 value of SJW was determined to be 50 µg / ml for 24 hours.

The aim of this study is to investigate the anti-cancer effects of SJW (hypericin) an in vivo experimental animal model of lung cancer.

Materials and Methods

This study was approved by Dokuz Eylul University Local Ethics Commitee for Multidisciplinary animal research by number 28/2019.

Nature's Bounty St. John's Worth: Hypericum perforatum (over earth), includes Hyipericin 0.9 mg (0.3%) in one capsule and Doxorubicin (KOÇAK) (10 mg/5 ml) are the chemicals used.

Cell Culture based in-vitro studies

Lewis Lung Carcinoma (LLC) (ATCC, CRl-1642) cell line was cultured in RPMI 1640 +10 % FBS+ 1 %Penicillin/Streptomycin + 1 %L-glutamin at 5% CO_2 37°C.

Doxorubicin (5mg/ kg) and SJW (5µg/ml-50mg/ml) doses were applied to 96 well plate with 6 wells of each condition at 24, 48 ve 72 hours. Proliferation was assessed with WST-1 at 450/630 by ELISA raeader [11]. Extracellular migration and invasion test were performed by invasion chamber in 24 well plate with polycarbonate membrane.

In Vivo Xenograft tests were performed using 35, 5-7 weeks old male nude mice average 25 grams in 5 groups (7 mice in each group) as follows:

1) Control group 0.9 cm tumor, (IP 0.3 cc saline)

2) Doxorubicin group (5mg/kg) (IP in 0.3 cc)

3) Group to examine the slowing effect of SJW (when tumor size reaches 0.2 cm, 50 ug SJW was applied)

4) To study the treatment effect of Group SJW (when tumor size reaches 0.9 cm, 50 ug SJW was applied)

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5) Doxorubicin (5mg/kg) and 50 ug SJW combination

The animals were kept in HEPA filtered cabinets at standard conditions (20 ± 2 ºC) room temperature and 12 hours day/night cycle. They were fed by sterile water and pellet ad abitum. After 5 days $4x10^6$ LLC cells in 0.3 cc RPMI were injected to left flanck. Daily observation, weight control, tumor diameter control was done till sacrification. When tumors reached to 0.2 and 0.9 cm in greatest diameter, they were randomized to groups.

After 7 days animals were sacrified under Halotan anestesia (Halotan BP 250 ml Pirimal). Whole blood from vena cava inferior and urine from the bladder were aspirated. Strip urine test was applied. The blood near 0.5 cc each was immediately centrifugated at 2000 x G in microtube. Supernatant serum was seperated and kept at -20 C till biochemistry. Serum glucose (mg/dL), creatinine (mg/dL), AST(U/L), ALT(U/L) was calculated by spectrophotometric analysis with IVD veterinary Preventive Care Profile Plus kit (ABAXIS, Germany) at Vetscan VS2 Chemistry Analyser (Abaxis) at Dokuz Eylul University Izmir Health Technologies Development and Accelerator Center (BioIzmir). Calibration was done by Abaxis Control-I (Randox) kit. Urine tests microalbumin and creatinine were done by IVD Clintek Microalbumin kit (SIEMENS, Germany) with Clintek Status Analyzer (Siemens) at BioIzmir. Check-stix Combo (SIEMENS, Germany) kit was used for calibration. Tumor tissue, lung, kidneys, heart, brain, liver were kept in 10% neutral formaline and then embedded in parafin. Tissue sections were stained with hematoxylin & eosin and TUNEL, Caspase 3,8,9, Ang1, Ang 2 immunohistochemistry (IHC) were performed to tumor tissues.

Apoptosis rate was determined by IHC. To do this, tissues were stained with Caspase-3, Caspase-8, and Caspase-9 proteins. Sections from cassettes obtained from tissues were first deparaffinized and treated with 3% H_2O_2. The washing step was carried out with distilled water and PBS for 10 min. Then tissues were treated Blocker A for 5 minutes and Blocker B for 5 minutes. The primary antibodies used were diluted 1:200 and the slides were treated with the antibody for 1 hour. After primary antibody, all slides were washed with PBS for 10 min. Secondary antibody was added and left for 30 min. After washing with PBS, DAB dye and H_2O_2 were added and left for 30 minutes. Slides were washed with tap water and PBS, respectively. Slides were treated with Copper D and waited for 4 minutes. Then, all slides were washed with tap water and stained with hematoxylin and eosin dye. Slides, which were washed again with tap water, were treated with a bluing reagent for 5 minutes. Then all slides were washed with tap water again. Finally, all slides were subjected to a series of increasing alcohol and treated with xylol for 1 hour. Before the xylol was completely dry, the slides were covered with a coverslip with Entellan and subjected to microscopy.

Results

WST-1 cell proliferation tests showed that LD50 for SJW is 50 µg/ml (Figure 1). When the experimental results were evaluated, it was observed that invasion and migration decreased significantly in 24 hours in the SJW group and 48 hours in the combined drug group. When SJW was applied to a 9mm tumor, no reduction in tumor size was observed (Figure 2). However, it caused necrotic cell death in tumor tissue (Figure 3). When comparing the control group with SJW,

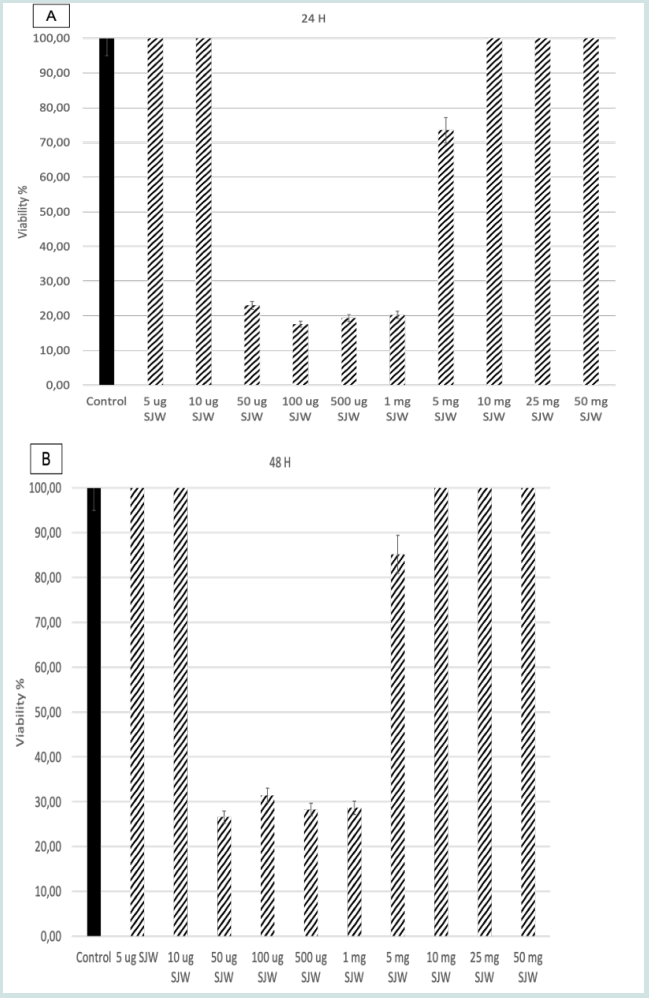

Figure 1: Cell viability results for SJW at a) 24h b) 48h c) 72 h incubation.

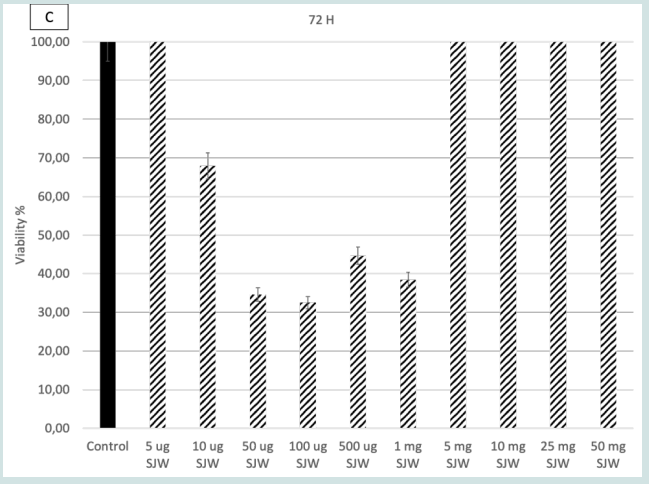

Figure 2: Tumor sizes of the 5 experimental groups. a) Control Group b) Doxorubicin Group. c) Group to examine the slowing effect of SJW (when tumor size reaches 0.2 cm, SJW will be applied) d) To study the treatment effect of Group SJW (when tumor size reaches 0.8 cm, SJW will be applied) e) Doxorubicin (5mg/kg) and SJW combination] before sacrification.

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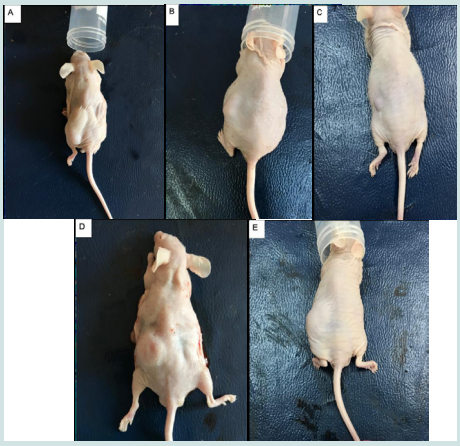

Figure 3: Histopathology of tumors: a) Control group: no necrosis; b) DOX group: prominent necrosis; c) SJW early: prominent necrosis cell death appears; d) SJW late: prominent necrosis cell death appears; e) DOX+SJW: prominent necrosis cell death appears.

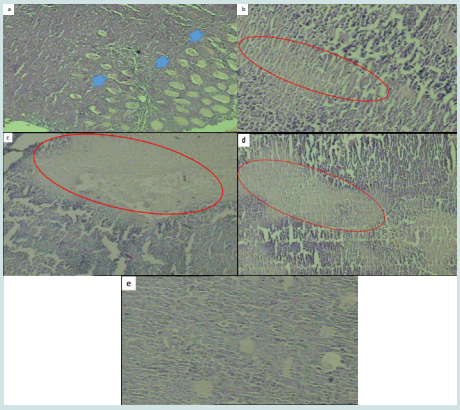

Figure 4: a). Viable tumour tissue in control mouse. The wide arrow represents invasive border. b). The red ellipse represents prominent necrosis area. c). The red ellipse represents prominent necrosis cell death area. d). The red ellipse represents prominent necrosis cell death area. e). All of the are represents necrotic tissue.

it was observed that tumor cell necrosis was statistically different in the SJW group. This necrotic effect was observed both on 9 mm tumors and when SJW was given after 0.5c2 mm tumor formation. Similar tumor necrosis was observed in the SJW + DOX group. However, no synergistic effect was observed in the combination of SJW with DOX. Although the apoptotic effect is higher in the combination group than the control group, the necrotic effect is lower than the control group.

Tunel Assay Results

The mean apoptosis ratio for all groups was 37.38% ± 5.701 (0-20). The mean apoptosis ratio was 3.20% ± 2.683 (0-6) in the control group, 6,00 % +- 4,183 (0-10) in the Doxorubicin group, 15.00 % +- 5.774 (10-20) in the late SJW group, 8.20% +- 6.834 (3-20) in the early SJW group and 6.00% +- 2.236 (5-10) in the combination group.

The highest apoptosis rate was observed in Late SJW group. Combination did not caused synergistic effect for apoptosis.

This apoptotic effect occurred both through intrinsic and extrinsic pathway. İmmunohistochemical parameters are given in Table 1. SJW application increased Ang1 expression slightly, while DOX application increased Ang1 expression more. Ang2 expression has not changed. In biochemical blood results only, AST levels increased in SJW + DOX group, while other test results are similar among the groups. As a result, SJW has been shown to have an anti-tumoral effect similar to DOX in the in-vivo experimental animal subcutaneous lung cancer model. No synergistic effect was observed in application with DOX.

Table 1: İmmunohistochemical Parameters

	Control (%)	Early SJW (%)	Late SJW (%)	Doxorubicin (%)	Combination (%)
Caspase 3	90	90	66.50	90	67.73
Caspase 8	13.33	22.50	7.14	50	10
Caspase 9	10	24.25	8.50	24.60	4.75
Ang 1	60	60	85.7	80	57.1
Ang 2	60	60	71.5	100	57

Discussion

The anticancer effect of SJW on a wide variety of cancer types has been studied. Liu et al. showed that hyperoside, one of the active ingredient of SJW, exerted inhibitory role in lung cancer development [9]. Yang et al. showed that hyperoside significantly inhibited the viability of lung cancer cells in a time- and dose-dependent manner and enhanced the percentage of apoptotic cells [10]. In our study, hypericin, another active ingredient of SJW, was studied. Hypericin photodynamic therapy (PDT) efficacy has been studied in a mouse tumor model. In the study, the primary mechanism of hypericin-mediated PDT mechanism stems from vasculature damage [8].

The mechanism of apoptotic process due to photodynamic therapy of hypericin in Jurkat cells has also been studied. The treatment also increases the activity of caspase-8 and caspase-3 and increases apoptosis, which can be blocked by caspase-8 (Z-IETD-FMK) and caspase-3 (Z-DEVDFMK) inhibitors [8]. SJW ethanol extract inhibited cell growth in a dose-dependent manner, as in ethanol extract, in an apoptosis-induced manner [7]. In addition, this SJW extract inhibits the AMPK / mTOR pathway, causing an increase in the expression of pro-apoptotic proteins BAX and BAD, and a decrease in the expression of anti-apoptotic proteins BCL-2, BCL-XL [7]. In cell leukemia lines (K562 and U937) made by Hostanska et al., Hypericin and hyperforin, which are the active ingredients of SJW, have synergistic effects, hyperforin induces apoptosis in doses, and proliferation of hyperforin (K562 and U937) [12]. In a device made by Stavropoulos et al., The effect of SJW, which has photostotoxic effect in cancer, on cell proliferation was investigated in vitro. In the study, when applied with 4-8 J / cm2 laser application on SJW cells in 60 ug / ml sul, the execution head inhibited cell proliferation more than 80% [13].

In the study of Borawska et al., It was found that SJW application in the Glioblastoma cell line (U87MG) inhibits proliferation and migration depending on the dose and time; and its anti-proliferative and anti-migration properties have been shown to increase synergistically with other herbal components such as propolis [14].

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In the study conducted by Mirmalek et al., SJW, which has a cytotoxic effect, was used in addition to Cisplatin in the MCF-7 breast cancer cell line to overcome the chemotherapy resistance seen in breast cancer. In the study, SJW has been shown to decrease cell proliferation in a dose and time dependent manner. It has been shown that SJW, whose LD50 dose is 5 µg / ml in 24 hours incubation, causes 60% apoptosis in MCF-7 cells. The fact that it causes an increase in BAX expression and a decrease in BCL-2 expression supports the apoptosis-inducing effect of SJW [15]. In the study of Dona et al., the anticancer properties of SJW in a wide variety of cell lines were investigated. Fibrosarcoma (HT-1080), neuroblastoma (SK-N-BE), Melanoma (B16-LU8), adenocarcinoma (c-26) cell lines were used in the study, and SJW was shown to inhibit cell proliferation in a dose-dependent manner (IC50 value is 5). It varies from cell to cell between 8 umol / l). As a result of the study, it was shown that hyperforin, the active ingredient of SJW, can be an effective herbal agent in preventing cancer invasion and metastasis [16].

The effects of SJW have also been examined in lung cancer, and the majority of studies have been conducted in-vitro. In studies using the non-small cell lung cancer cell line A549, it has been shown that the active ingredients of St. John's wort, hyperforin and hypericin, induce autophagy and apoptosis, regulate caspase-3, caspas-9, and p53-related cell death, depending on dose and time [17-19]. All these results show that SJW extract has anti-proliferative and pro-apoptotic effects.

Conclusion

In conclusion SJW is shown to have antitumoral effect in subcutanous xenograft lung cancer in vivo model in nude mice. Dose was obtained comparing with DOX. In combination with DOX, there were no synertistic increase in anti-tumor effect. SJW might be a candidate antineoplastic supplementation in lung cancer. Since this is an acute study, the treatment response to the agent administered within the first 7 days is interpreted by agent-induced cell death within the tumor rather than reduction in tumor size.

Acknowledgement

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All animal experiments comply with the ARRIVE guidelines and carried out in EU Directive 2010/63/EU for animal experiments.

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