ISSN NO. 2320-5407

Journal homepage:http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Apoptotic and anti-angiogenic activity of *Hypericumperforatum*L on 4-(methylnitrosamino)- 1-(3-pyridyl)-1-butanone-induced lung cancer in rats

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Manuscript Info

Manuscript History:

Received: 12 November 2013
Final Accepted: 19 December 2013
Published Online: January 2014

Key words:
Hypericumperforatum- CD31- lung- NNK- cancer. MVD- angiogenesis

Abstract

Purpose, St. John's wort (*Hypericumperforatum*L.) is widely known as an herbal medicine used to treat anxiety, depression and wounds but has not been tested as lung cancer preventives yet. In the present study, the efficacy of *Hypericumperforatum* against rat lung cancer has been investigated. Methods, a total of 60 adult male Sprague-Dawley rats were randomly divided into four groups. Group 1 rats were given normal saline. Group 2 rats were given nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) with two alternate doses (100mg/kg/bw). Group 3 rats were given NNK+ *Hypericumperforatum* (300 mg/kg/bw).Group 4 rats were fed with *Hypericumperforatum* only (300 mg/kg/bw). Results; it was found that the dietary supplementation could able to reduce lung tumor prevalence by 80%. Conclusion, it is suggested that *Hypericumperforatum* exhibit anticancer activity through its attribution to the actions of inhibiting growth, metastasis and neovascularization.

Introduction

Tobacco smoking is an enormous environmental exposure. It has been estimated that there are 1200 million smokers throughout the world. Globally it is estimated that 47% of men and 12% of women smokes (World Health Organization 1997).Epidemiological studies have indicated that cigarette-smoking habits are associated with a higher incidence of lung cancer (Videtic et al., 2003). Tobacco-related cancer constitutes 16% of the total annual incidence of cancer cases-and 30% of cancer-related deaths in the developed countries. Although tobacco smoke contains more than 3000 chemical compounds; it has been proposed that nicotine is one of the key constituents causing adverse health effects and nicotine metabolic products may also be harmful (Benowitz, 1986).

At least 60 chemicals, including nitrosamines are proven to be carcinogenic. Among them NNK, a tobacco-specific N-nitrosamine, is believed to be one of the most promising candidates of lung carcinogen in mice, rats, and hamsters (Hecht, 1999). It is suggested that NNK is strongly related to human pulmonary adenocarcinoma (Yang, 2002). An etiological role of NNK in the induction of human lung cancer is supported by evidence showing that NNK is activated to form DNA-damaging agent in human lung tissue, and that the lung is an important target organ for NNK-induced carcinogenesis in rodents (Castonguay et al., 1993).

Lung cancer is the most common cancer and leading cause of cancer death globally. This high mortality rate is, first of all, due to the fact since most cases of lung cancer are diagnosed at an advanced stage of development. Despite all the great efforts to improve smoking cessation and the treatment of patients with lung cancer, the survival rate for people diagnosed with this disease has not significantly improved over the past 30 years (Pankiewicz et al., 2007). And most of the deaths from lung cancer are due to smoking tobacco products (Ezzati& Lopez, 2003).

The tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) forms during the curing and processing of tobacco by the nitrosation of nicotine. NNK is present in cigarette smoke. It is a potent carcinogen in laboratory animals, and its specificity for the lung has implicated it as an etiological agent of lung cancer in smokers (Hecht &Hoffmann, 1989).

NNK is a selective inducer of adenocarcinoma of the lung in rodents. In F344 rats, lung tumors were induced regardless of the route of administration, including in drinking water, or by subcutaneous, gavage, oral swabbing or intravascular injection. (Zheng et al., 2002). Over the last decades, natural compounds have attracted considerable attention as cancer chemopreventive agents and as cancer therapeutics. Among their various biological activities, natural products can modulate apoptosis signaling pathways (Fulda, 2010).

The use of herbal medicinal products is rapidly growing, and one of the most popular herbal remedies is St. John's wort, consisting of the leaves and the flowering top of *Hypericumperforatum*. St. John's wort (Hypericumperforatum L.) is a member of the hypericaceae plant family. Recently, it has gained a reputation as an effective treatment for depression in most countries (Lindeet al., 1996; Woelk 2000; and Jain et al., 2010). One of the active ingredients of St. John's wort is Hyperforin. It is a natural antibiotic that inhibits the growth of several gram-positive bacteria, including meticillin resistant *Staphylococcus aureus*. In a previous study of Schempp and hiscolleagues (Schemppet al., 2000), observed a dose-dependent antiproliferative effect of hyperforin in PHA-stimulated peripheral blood lymphocytes, by induction of enzymes of the cytochrome P450 system (Moore, et al., 2000)as well as P-glycoprotein (Hennessy, et al., 2002). Besides the previous report; that hyperforin-related substances (hyperevolutins) inhibit the growth of a colon carcinoma cell line (Schempp, et al., 2002).

Presently, there are no experimental studies to support the effect of hyperciumperforatum on lung tumorigenesis and the mechanism(s) of action involved. This study was undertaken to evaluate the ability of hyperciumperforatum to inhibit NNK- induced lung cancer in rats, the modulatory effects of the *Hypericumperforatum* on neovascularization (MVD), assessed by the analysis of CD31 immunoreactivity, in the lung lesions induced by NNK, and whether *Hypericumperforatum* displays antiproliferative effects on tumor cells.

Materials and Methods

Animals:

Sixty adult male Sprague-Dawley rats, aged about 16 weeks of age and weighting 200± 20g were housed in a temperature-controlled environment (25 °C) with 12-h light-dark cycle. Food and water were freely available. The experiments were conducted in accordance with national animal protection guidelines and performed at the animal house in the genetic Engineering and Biotechnology Inst. Menofia University.

Chemicals

NNK 4-(methylnitrosamine)-1-(3pyridyl)-1-butanone was purchased from Sigma-Aldrich Co.

Hypericumperforatum extract (St. Johns wort)(Vital Nutrients).

Tumor induction:

Rats were injected intraperitoneally twice with NKK (100mg/kg/b.w. in 0.1 ml PBS) on two alternate weeks. Control animals received PBS. A total of 60 rats were randomly assigned to 4 groups (n = 15 animals/ group).

Group 1: (control group) rats were received normal saline intraperitoneally.

Group 2: rats were given NNK with two alternate doses (100mg/kg/bw).

Group 3: rats were given NNK+ *Hypericumperforatum* (300 mg/kg/bw).

Group 4: rats were fed with *Hypericumperforatum* only (300 mg/kg/bw) orally (using gavage) as a watery slurry at a daily dose for 16 weeks.

Tissue collection

After the treatment period (Sixteen weeks following NNK injection to allow time for adequate tumor development) all rats were sacrificed under ether anesthesia. Lungs were examined by naked eye to make sure there is lung mass or not, then lungs were removed, washed several times with physiological saline, and were fixed by intratracheal instillation of 10% neutral buffered formalin. The preserved tissues were dehydrated, embedded in paraffin, sectioned at 3-4 um thickness, and stained with H&E; microscopic examination was performed to histopathologically investigate proliferating lesions in the lung tissue.

Immunohistochemical staining

Immunohistochemical detection of CD31

Tumor tissues were fixed immediately in 10% neutral buffered formalin and embedded in paraffin. Immunohistochemical staining was performed using a labeled streptavidin-biotin method. Briefly, the dewaxed, rehydrated sections (5μm) were treated with 0.3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity and then washed three times with phosphate-buffered saline (PBS, pH 7.2). The sections were boiled with ethylene diamine tetra acetic acid (EDTA) buffer solution (pH 8.0) for 20 min. with microwave, followed by cooling at room temperature for 20 min. Nonspecific binding was blocked with 2% normal goat serum at room temperature for 15 min. followed by treatment with primary antibody at 37°C for (anti-CD31 diluted for

1:100) BD Pharmingen for one hour at room temperature. After washing by PBS, sections were then incubated with biotin labeled secondary antibody for 10 min and washed with PBS. Peroxidase conjugated streptavidin was added for 20 min and then washed with PBS. Finally, sections were developed with 3, 3-diaminobenzidine and hydrogen peroxide (DAB substrate kit) as chromogen and counterstained with hematoxylin. For the negative control, PBS was used instead of primary antibody. The sections were analyzed by light microscopy.

Detection of microvessel density (MVD)

MVD was assessed through immunohistochemical analysis with antibody to the endothelial marker CD31 and determined according to the method of (Weidner et al., 1991). Briefly, the immunostaining sections were initially screened at low magnifications (40× and 100×) to identify hot spots (areas with the highest microvessel concentration), which are the areas of highest neovascularization. Any yellow-brown stained endothelial cell or endothelial cell cluster that was clearly separated from adjacent microvessels, tumor cells, and other connective tissue elements were considered a single, countable microvessel. Within the hot spot area, the stained microvessels were counted in a single high-power (200×) field, and the average vessel count in three hot spots was considered the value of MVD.

DNA fragmentation

Nucleic acids extraction and molecular assessment was based on salting out extraction method (Aljanabi, & Martinez, 1997). For apoptosis, the extracted DNA was gently resuspended with TE buffer supplemented with 5% glycerol, gently pipetting, then the samples were mixed with 6 × loading buffer and loaded directly on to gel (Hassab El-Nabi, 2004). The remaining DNA was kept at –20°C for another loading. Apoptotic bands appeared and were located at 200 bp; 400bp; 600 bp and 800 bp. The intensity of apoptotic bands and intact DNA could be electrophoretically separated on 2% agarose gel containing 1 µg /ml ethidium bromide and visualized under ultraviolet transillumination.

Z score was used to get p value, p value of 0.01 was considered statistically significant.

$$z = \frac{\hat{p}_1 - \hat{p}_2}{\sqrt{\hat{p}(1-\hat{p})\left(\frac{1}{n_1} + \frac{1}{n_2}\right)}}$$

Results

Gross evaluations

Gross evaluation revealed no tumor formation were observed in the control rat lungs (group 1), and *Hypericumperforatum* treated rats (group 4). Conversely, administration of a double dose (two alternate doses) of NNK to rats in group 2 produced a lung tumor incidence in all NNK-treated rats (100%) with heterogeneity in the morphological appearance of the neoplastic lesions was also observed. And in group 3 treated with NNK plus *Hypericumperforatum* revealed inhibition for tumor growth by 80% (12/15).

Histological observations

The histologic examination of lung sections was focused on proliferative lesions. No histologic evidence of premalignant or malignant neoplasms was found within the control group or *Hypericumperforatum* treated group. On the other hand,Light microscopic examination ofrats treated with NNK plus *Hypericumperforatum* showed noticeably obvious signs of protection in lung tissue by where, reduction of lung cancer mass, appearance of inflammatory alterations and hyperplasia in addition to normal histology strikingly similar to that of the control group were also noted, there was statistical significance different compared to group2. Conversely, rats that treatment with NNK alone produces 100% (15/15) lung tumor incidence (Table 1). The histopathological analysis results confirmed the gross observation of lung tumor analysis results (Figure 1).

Immunohistochemical observations

MVD was determined by counting the number of the microvessels per high-power field (hpf) in the lung section with antibody reactive to CD31 compared with the control group. Rats in group 2 that injected with NNK alone revealed increasingly higher MVD counts in lung tissue (the positive expression for CD31) than those of rats from the control and *Hypericumperforatum* plus NNK (The average MVD counts, in NNK group, NNK plus hypericumperforatum group, *Hypericumperforatum* group, and control group were 78.1 (range, 50-166), 21.3

(range, 22-67), 9 (range, 7-13), and 8 (5-11), respectively. This difference was statistically significant ($P < 0.04$). The results indicated that *Hypericumperforatum* has anti-angiogenic effect and inhibited tumor angiogenesis, which play an important role for the treatment of lung cancer (Figure 2).

Apoptotic DNA fragmentation

Endonucleases and DNA fragmentation factors are activated during apoptosis, resulting in degradation of chromatin DNA at sites between nucleosomes units. (Protein-containing structures occur in chromatin at ~200-BP intervals).In the present study, it was observed that *Hypericumperforatum* induced apoptotic DNA fragmentation at 180 to 200 bp or multiples in lung cancer tissues (Figure 3), *Hypericumperforatum* administration resulted in significant induced apoptotic rates in lung tissue rats of group 3 (hypericumperforatum plus NNK) in case of control and *Hypericumperforatum* groups; no fragmentation was seen. As a result, rats treated with *Hypericumperforatum* suppressed tumor growth.

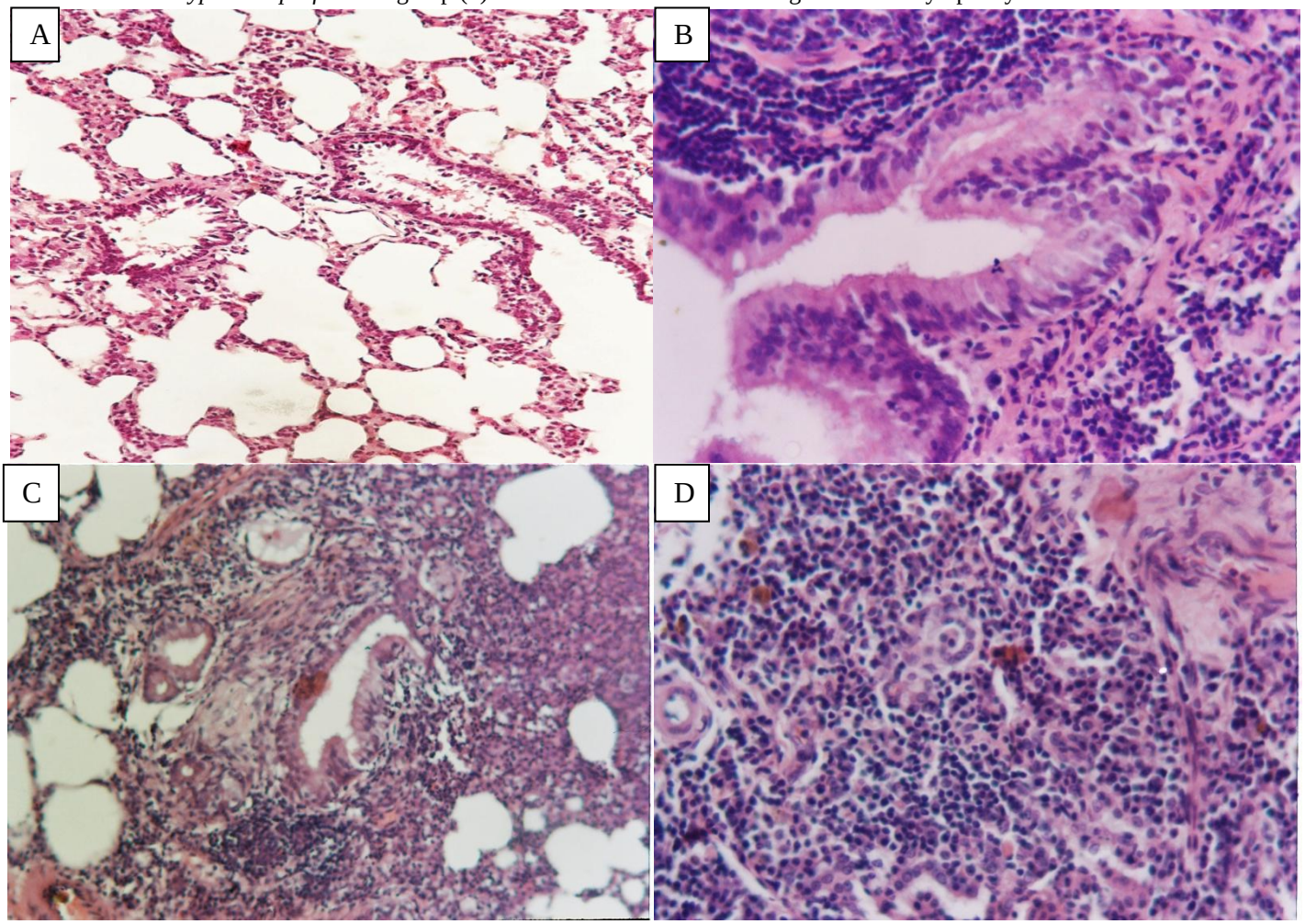
Figure 1: Photomicrographs of proliferative lung lesions in NNK-treated rats.

A- Control animal group treated with normal saline.

B &C- NNK + *Hypericumperforatum* group (3) showed hyperplasia in epithelial lining and inflammation reaction. Chronic inflammation reaction with fibrous connective tissue and giant alveolar cells were also shown, respectively.

D & E - NNK group (2) showed adenocarcinoma with active cell hyperchromatism. And tumor cells were metastasis in blood vessel.

F –*Hypericumperforatum* group (4) shown normal structure of lung tissue with lymphocytic infiltration.



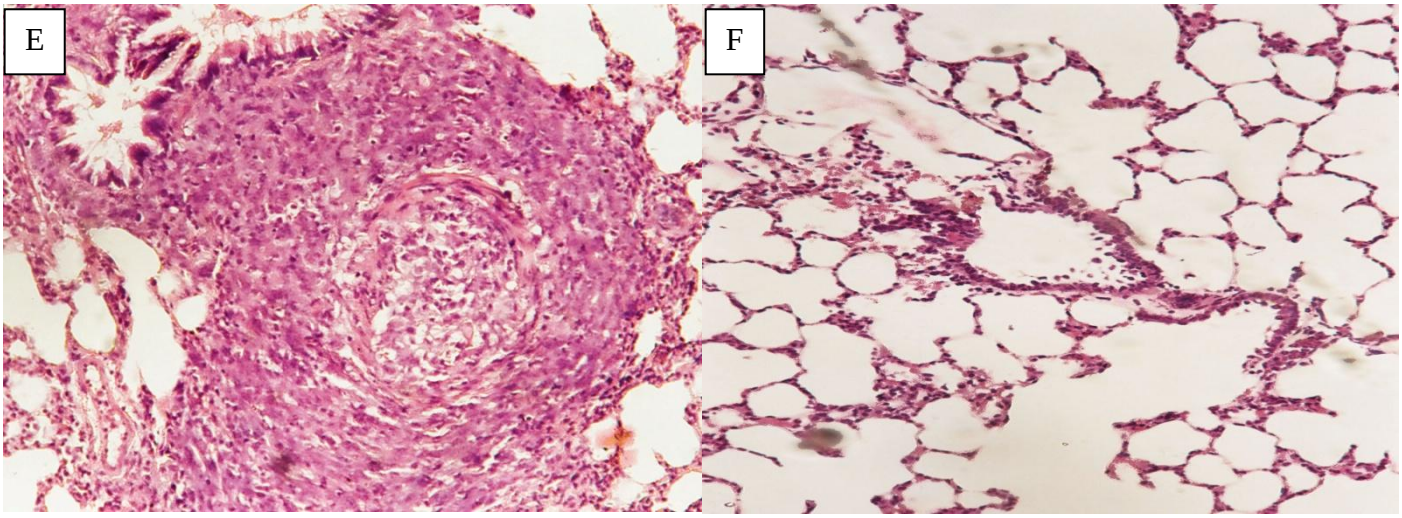
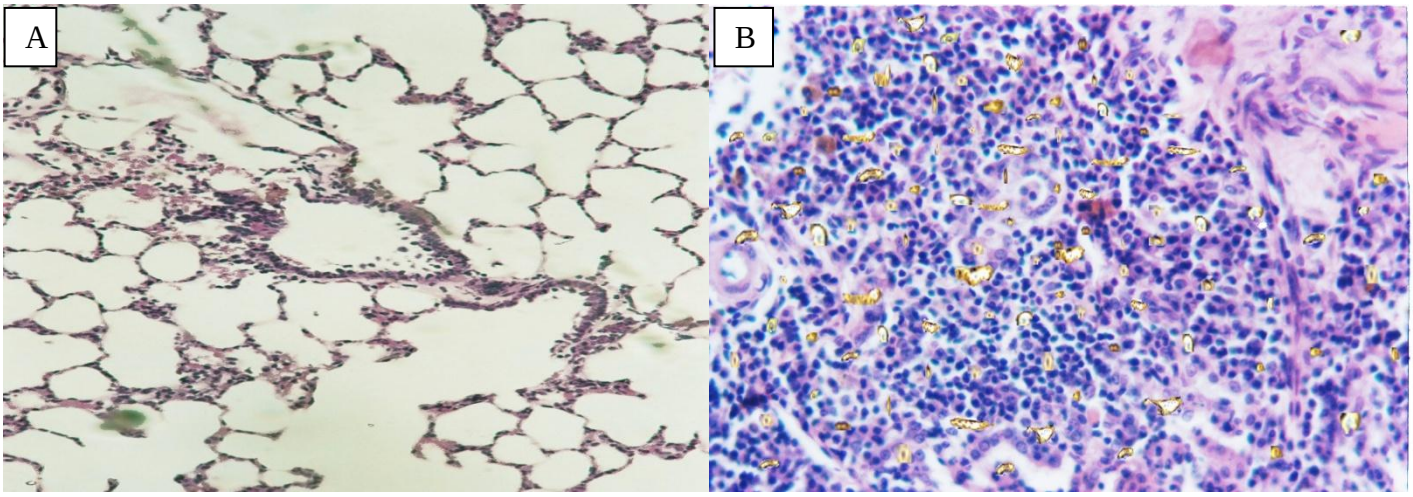


Figure 2: Photomicrographs of immunohistochemical examination of the expression of CD31 in lung lesions induced by NNK in rats.

- **A-** Normal lung epithelial cells showed negative expression of CD31
- **B-** Expression of CD31 in lung cancer induced by NNK showed high expression of a lot of vessel endothelial cells.
- **C-** Reduced expression of CD31 in lung tissues of group 3



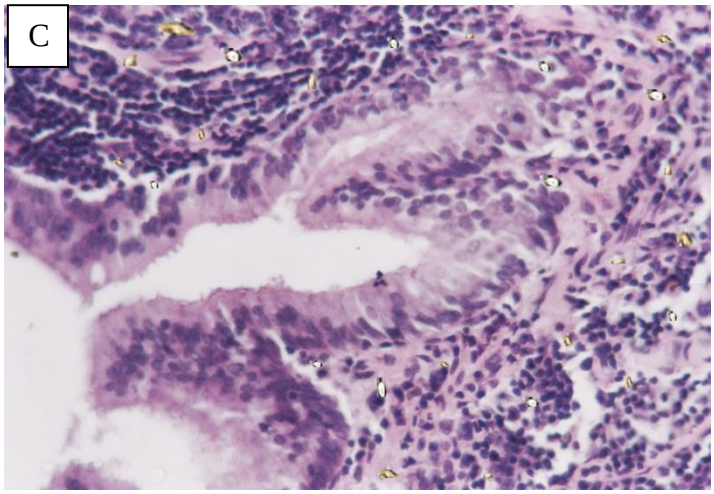


Figure 3: Apoptotic DNA fragmentation of lung tissue rats. Lane M: ladder; lane 1: control group; lane 2: NNK treated group; lane 3: NNK plus *Hypericumperforatum* group; lane 4: *Hypericumperforatum* group.

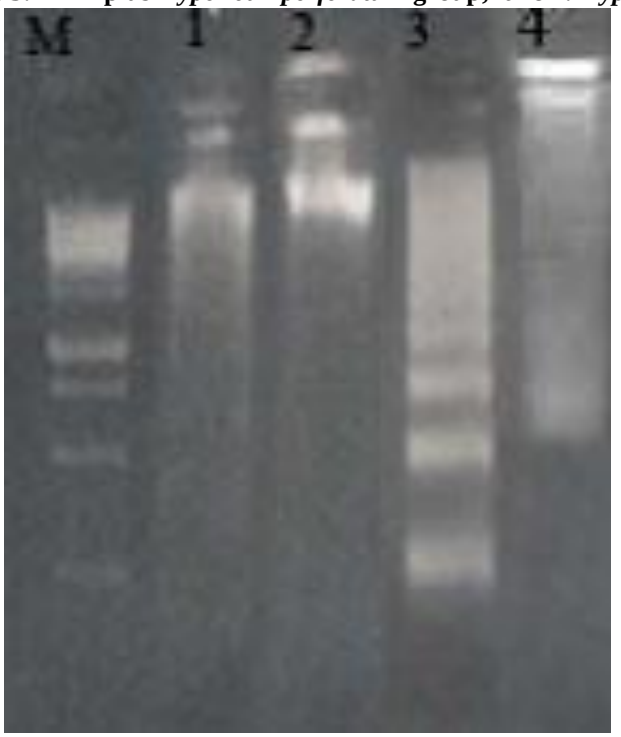


Table 1: The effect of *Hypericumperforatum* on lung tumorgenecity in rats treated with NNK.

Table1

group	no. of rats	Tumor incidence		Tumor inhibition	
		no.	%	no.	%
Control	15	0	0	0	0
NNK	15	15	100	0	0
NNK+ HP	15	3	20	12	80
HP	15	0	0	0	0

Statistical difference was observed between NNK and NNK+HP groups (Z= 24.76), $p<0.01$
Statistical significance was tested by using Z score.

Discussion

High mortality because of the lung cancer comes out mainly of the fact that majorities of the cases are diagnosed at advanced stages. An expanded diagnostic means of pre-invasive conditions, which is referred to as precancerous lesions, would certainly contribute to lower the motility rate (Pankiewicz, et al., 2008). Over the last decades, natural compounds have attracted considerable attention as cancer chemopreventive agents and as cancer therapeutics (Nabili et al., 2009). Among their various biological activities, natural products can modulate apoptosis signaling pathways.

Carcinogens such as NNK require metabolic activation to exert their carcinogenic effects; there are competing detoxification pathways, and the balance between metabolic activation and detoxification differs among individuals and will affect cancer risk (Ye et al., 2007).

We question whether the inhibition of tumor cells by *Hypericumperforatum* was associated with the induction of apoptosis. The content of apoptotic oligonucleosomes was evaluated by DNA fragmentation. DNA extracted from lung tissues of rats, which injected with NNK and treated with *Hypericumperforatum* (group 3)

shown an increase of apoptotic DNA fragments as compared to other groups. And this result was in agreement among the results reported by (Schempp et al., 2002). And also results are in harmony with data obtained by (Coussens et al., 2002), who has found that Hypericumperforatum shows anti-proliferative effect on tumor cells in vitro, (Di Carlo et al., 2003) demonstrated that *Hypericumperforatum* was found to inhibit the growth of, and induce apoptosis in, mouse tumor models and that this action could be mediated in part by an increase in Bcl-2 expression.

It was suggested that treatment with HP induces apoptotic DNA fragmentation by stimulating the mitochondrial apoptosis pathway (cause mitochondrial outer membrane permeabilization and cytochrome c release in a Bcl-2 or Bcl-X dependent manner (Fulda, 2010), and results in suppression of tumor formation. Another suggestion reported by (Dell'Aica et al., 2007) that *Hypericumperforatum* is a hyperforin. It acts via inhibition of the reuptake of serotonin and other neuro-transmitters back to the synapses and blocks tumor cell growth by inducing mitochondrial permeabilization-triggered apoptosis. Consistent with these finding, DNA fragmentations (apoptosis) was induced in the lungs of rats treated by *Hypericumperforatum*, this finding supported by previous report (Wang et al., 2001).

Our results confirmed that 300 mg/kg body weight of *Hypericumperforatum* per day significantly reduced CD 31 expressions in lung tissue, thus, indicating that neo-vascularization is entirely inhibited and this may be attributable to its activity in prevention of metastasis. According to the present data of CD31 staining, it was observed that the MVD of the *Hypericumperforatum* plus NNK group obviously lessen compared with the control and NNK groups. We assume that *Hypericumperforatum* administration inhibited tumor neovascularization either by limiting early VEGF production and/or by targeting the expression of other proangiogenic mediators, these findings are in line with previous research report (Donà et al., 2004), who used two murine experimental metastasis models,in which syngenic C-26 colon carcinoma or B16-lus melanoma cells injected I.V. in animals receiving i.p. *Hypericumperforatum* twice /day, they found a significant reduction of pulmonary metastasis in comparison to the untreated group. Moreover, (Martínez-Poveda et al., 2010) reported that *Hypericumperforatum* had the potential of reducing invasion and metastasis.

The protective benefits of HP may be due in part to its potent antioxidant properties and ability to reduce oxidative stress. Treatment with the HP overpowers the onslaught by suppressing the formation of reactive oxygen species and protecting the antioxidant mechanism. And our findings were in agreement with the previous data reported by (Bitiren et al., 2010). *Hypericumperforatum* exhibit anti-cancer activity and its effect has been attributed to the actions of inhibiting growth, metastasis, and neovascularization. The protection against cancer may arise from several mechanisms, including inhibition of the formation of chemical carcinogen, decreased of activation and /or increase of detoxification of carcinogens (Schempp et al., 2000), induction of cell cycle arrest (Meruelo et al., 1988), and apoptosis (Krusekopf, & Roots, 2005). In conclusion, it has been established that several components of *Hypericumperforatum* inhibit a variety of tumor pathogenesis processes in lung tissue in the experimental model.

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