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Observations on the Biological Effects of Black Cumin Seed (*Nigella sativa*) and Green Tea (*Camellia sinensis*)

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Abstract: The biological effect of Black Cumin seed (*Nigella sativa*; Ns) and Green tea (*Camellia sinensis*; GT) has been tested using antiviral, anti-cancer, anti-angiogenic and antioxidant assays. Results showed that Ns and GT have antiviral activity against Infectious Laryngotrachietis Virus (ILTV) at concentration of 35 and 4.22 µM, respectively. Also, both plants protected rats from Diethylnitrosamine (DEN) induced hepatocellular adenoma. Moreover, Ns showed inhibition rate of 80 and 65% at concentration of 80µM, while GT showed respective values of 75 and 45% and 90 µM on Hella and Vero cells. Ns and GT showed marked anti-angiogenic activity on endothelial cells of rat's aorta as well as antioxidant activity by Diphenyl Picryl Hydrazyl (DPPH) radical scavenging activity, Nitric Oxide (NO) radical inhibition assay and lipid peroxidation assay. Current data augmented the efficacy of *Nigilla sativa* and green tea as remedies for viral diseases, cancer, angiogenetic disorders and oxidative stress.

Key word: Antiviral . anti-oxidant . anticancer . angiogenesis . Nigella sativa . green tea

INTRODUCTION

Nowadays, there are increasing trends to use herbal life style and dietary choices for human welfare and to improve the productivity and health of farm animals. These natural products can help the whole body and improve the immunological status. It was reported that Ns improves the average daily gain, feed digestibility and nutritive value, have immunostimulant and hepatoprotective effects and improve the reproductive performance and thyroid function in farm animals [1, 2].

Ns and GT have immunostimulant effect and used as remedies for cancer and diseases, along with other plant extracts in traditional medicine, with variant degree of success. However, until recently, none of these preparations have been subjected to any form of controlled scientific investigations to evaluate their efficacy as curative agents for cancer or viral diseases. NS has been used as antiviral agent against murine cytomegalovirus infection [3]. Also, it has been used as anti-hepatocarcinogenic agent [4]. While, GT has been used as anti-viral agent against influenza [5], adeno [6] and herpes simplex type 2 [7] viruses. Moreover, it has given satisfactory results when applied for prevention of liver cancer in human [8].

Free radicals have aroused significant interest among scientists in the past decade. Their broad range of effects in biological systems has drawn the attention of many experimental works. It has been proved that their mechanism of action is an important factor in the pathogenesis of certain diseases and ageing process. There are many reports that support the use of antioxidant supplementation for reducing the level of oxidative stress and in slowing or preventing the development of complications associated with diseases. Many synthetic antioxidants have proven to show free radical scavenging or antioxidants activity [9]. In the same time, flavonoids and other phenolic compounds of plants origin have been reported as scavengers and inhibitors of lipid peroxidation [10].

The current investigation was designed to monitor the biological effects of some commonly used plants (*Nigella sativa* and green tea) from the anti-viral, cancer, angiogenesis and oxidant points of view.

MATERIALS AND METHODS

The present investigations were carried out at the National Research Centre, Egypt and University of Mentouri, Algeria during the period from March to December, 2007.

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Experimental materials

Plant material: The black cumin seeds *(Nigella sativa*; Ns) and leaves of green tea (*Camellia sinensis*; GT) were purchased from the local Egyptian market.

Cells: Hella and Vero cells were used for detection of anticancer effect. While Chicken Embryo Rough Cells (CER) were used in detection of antiviral effect against ILTV. Cells were purchased from VACSERA Institute, Agoza, Egypt.

Rats: Mature male rats (200-250g) were purchased from the Animal House of the National Research Centre for testing anticancer activity and for obtaining aorta for tissue culture.

Virus: Local isolate of Infectious Laryngotracheitis virus (ILTV), [11] was used to test the antiviral effect of both Ns and GT.

Chemicals and media: Chemical and media used in this work were obtained from Sigma Diagnostics *Inc. USA*.

Experimental design

Preparation of aqueous extract of Ns and GT: 500g of each plant were washed and boiled individually in distilled water for 1 hr. The resultant material was lyophilized and used then after.

Cytotoxicity assays: The cytotoxic effect of Ns on CER cells was performed as outlined by [12] and was recorded under the light inverted microscope. The Maximal Non-Cytotoxic Concentration (MNCC) was determined as the maximal concentration of the sample that did not exert toxic effect as detected by microscopic monitoring after 72 hrs of incubation.

Virus titration: The ILTV was titrated according to [13] and expressed as 50% tissue culture infectious dose/ml ($TCID_{50}$/ml).

Antiviral assay: It was done by Cytopathic Effect (CPE) method. The antiviral activity of Ns and GT extracts against ILTV were measured by CPE-reduction assay. CER cell cultures were prepared in 96-well microtitre plate (Falcon Plastics, Oxnard, CA, USA). After 24 hrs of incubation at 37°C in a 5% $C0_2$ incubator, the culture medium was removed from the monolayer cells. To confluent mono layers of CER cells in 96-well microtitre plate, 0.1 ml of ILT suspension containing 100 times of TC1D50 and 0.1 ml of maintenance medium containing an appropriate serially diluted concentrations of the test samples were added. MNCC of the test samples were used as the highest concentration (MNCC for Ns and GT which were 80and 90µM, respectively) from which a serial two-fold dilution was made with the culture medium [14, 15]. Cell controls and virus controls were run simultaneously. The plates were incubated at 37°C with 5% CO_2 usually for 4-5 days until the virus in the control wells showed complete virus-induced CPE as observed under light microscope. The concentration of the samples required to inhibit 50% of the growth of ILTV (IC_{50}) was estimated from the graphic plots [16]. The virus-induced CPE was scored on day 3 after infection. The reduction of virus multiplication was calculated as percentage of virus control (reduction of virus multiplication = $CPE_{exp}/CPE_{virus\ control}$ X100%). The concentration reducing CPE by 50% in respect to virus control at day 3 was evaluated from graph plots and was defined as 50% effective concentration (3 day's EC_{50}) according to [17].

Anticancer assay

***In vivo* anticancer assay:** 30 rates, were divided into six equal groups. Group 1, 2 and 3 were injected with 200mg/Kg Diethylnitrosamine (DEN) to initiate hepatocarcenogenesis [18], while group 6 was given normal saline i.p 24 hrs later, whole crushed dried GT leaves were given orally to groups 3 and 5 while, crushed Ns seeds were given orally to groups 2 and 4. Group 6 was left as a negative control. The oral feeding of the two plants continued for 3 months *ad labium*. At the end of this period, rats were sacrificed and livers were examined for tumor like lesions (TL). At necropsy, livers were excised and sliced into 2-3 mm thick (three slices of liver, one each from the right posterior, right anterior and caudate lobes) were cut with surgical blade, fixed in 10 phosphate buffered formalin. The samples were histologically processed and paraffin blocks were sectioned using a microtome and stained by Haemotoxylin and Eosin (H&E) according to [19].

***In vitro* anticancer assay:** The *in vitro* anti-cancer effect against Hella and Vero cell lines were evaluated by Trypan blue exclusion method by [20]. Hella and Vero cells were grown in sterile DMEM (Dulbecco's modified Eagle's medium) and MEM (modified Eagle's medium), respectively. Both media contained 0.2% sodium bicarbonate, antibiotics (1:1000 Fugizone and 1:100 Garamycine) and 10% heat-inactivated fetal calf serum (FCS). The cell cultures were incubated in full humidified atmosphere of 95% with 5% Co_2 at 37^o C. Haemocytometer was used to estimate the total number of viable and non-viable cells (by counting cells in the four 1 mm^2 corners of the haemocytometer) and

average number of the cells per unit volume (millimeter, ml) of medium was calculated as the sum of the counted cell number/ $4X10^4$. For determination of effective concentration for inhibition of cell proliferation, both cells were incubated with tested plant samples in multiple doses. The concentration of the samples required to inhibit 50% of the proliferation of the cell lines (IC_{50}) was estimated from the graphical interpolation.

Anti-angiogenic effect of *Nigella sativa* and green tea: 4 rates were sacrificed and their aorta was dissected under complete aseptic condition. The aortae of the four rats were cut into small cross sectioned pieces and used in tissue culture of aorta. The tissue culture was supplemented with Eagl's Minimal Essential Medium (EMEM) and 10% FCS and 1:100 Garamycin. The small pieces of aorta were propagated on 12-well tissue culture plate and the wells were divided into three parts. The first part contained cross sectioned aorta, EMEM and 2% FCS with Green tea. While the second part contained the same ingredients with Ns instead of GT and the last part contained negative control (crossly sectioned aorta supplemented with EMEM and 2% FCS and 1:100 Garamycin. The plates were daily examined under inverted microscope [21].

Antioxidant assay

DPPH radical scavenging assay: The free radical scavenging capacity of the two extracts was determined using 1, 1-diphenyl, 2-picryl hydrazyl (DPPH) as outlined by [22]. A methanol DPPH solution (0.15%) was mixed with serial dilutions (0.5 to 8µg) of both extracts and after 10 minutes, the absorbance was detected at 515 nm using a spectrophotometer. Vitamin C was used as a standard. The inhibition curve was plotted and IC_{50} values obtained.

Nitric oxide radical inhibition assay: NO radical inhibition was estimated by the use of Griess Illosvoy reaction [23]. In this investigation, Griess Illosvoy reagent was modified by using naphthyl ethylene diamine dihydrochloride (0.1% w/v) instead of 1-naphthylamine (5%). The reaction mixture (3 ml) containing sodium nitroprusside (10mM, 2ml), phosphate buffer saline (0.5ml) and both plant extracts each individually (10 to 160 µg) or standard solution (rutin, 0.5 ml) was incubated at 25°C for 150 minutes. After incubation, 0.5 ml of the reaction mixture mixed with 1ml sulfanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 minutes for complete diazotization. Then 1ml of naphthyl ethylene diamine dihydrochloride was added, mixed and allowed to stand for 30 minutes at 25°C. A pink colored chromophore is formed in diffused light. The absorbance of these solutions was measured at 540 nm using rutin as a standard.

Lipid peroxidation assay: The rate liver microsomal fraction was prepared by the method of [24]. The reaction mixture contained in a final volume of 1.0ml, 500µl of liver microsomal fraction, 300µl buffer containing the two plant extracts each individually (50-150µg), 100µl of $FeCl_3$ (1mM) and 100µl ascorbic acid (1 mM) to start peroxidation. Samples were incubated at 37ºC for 1 hr, after that lipid peroxidation was measured using reaction with thiobarbituric acid (TBA). TBA reactive substance was determined by the methods of [25]. The absorbance of the organic layer was measured at 532nm. All reactions were carried out in triplicate and vitamin E was used as standard.

RESULTS

The current studies revealed that both Nigella sativa and green tea have clear biological activities including:

Antiviral activity: *Nigella sativa* and green tea have antiviral activity against ILTV with EC_{50} of 35and 4.22 µM, respectively (Fig. 1).

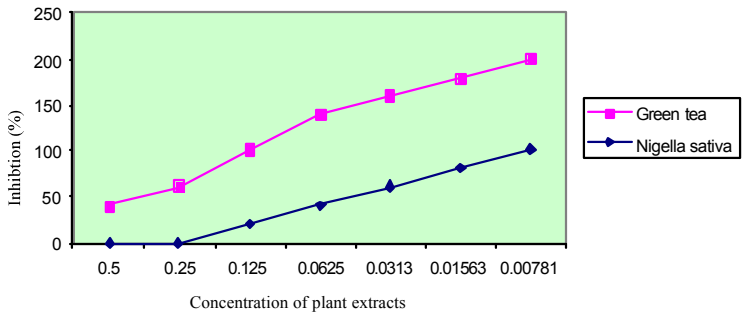
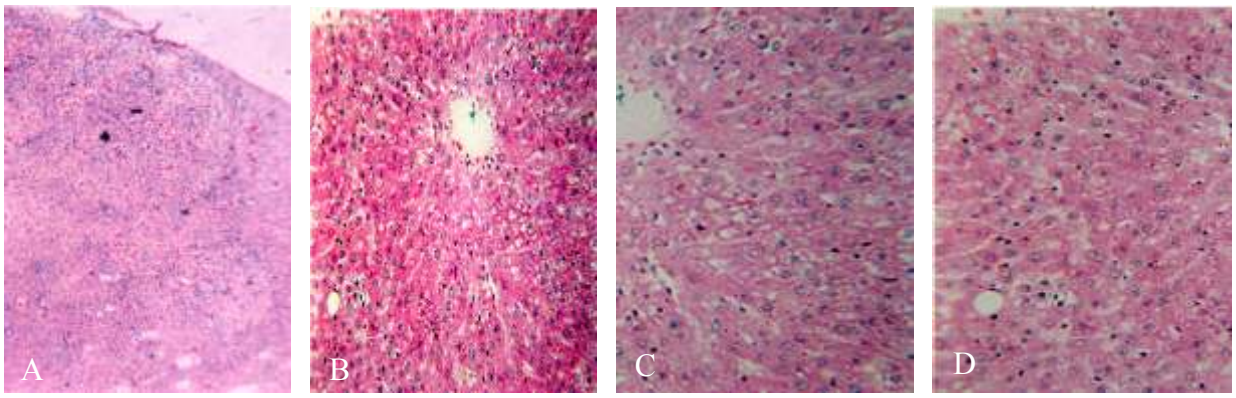


Fig. 1: Antiviral activity of *Nigella sativa* and green tea



Microphotograph 1: Liver sections of rats used for *in vivo* assaying of anti cancer activity, rat A given DEC, show hepatocellular adenoma, while, rats B given DEC+ NS, C, DEC+GT and D, Saline showed normal livers (H&E, X= 100)

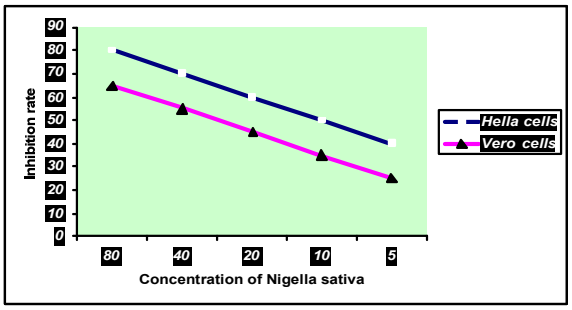


Fig. 2: Anti-cancer effect of *Nigella sativa* aqueous extract on Hella and Vero cell lines

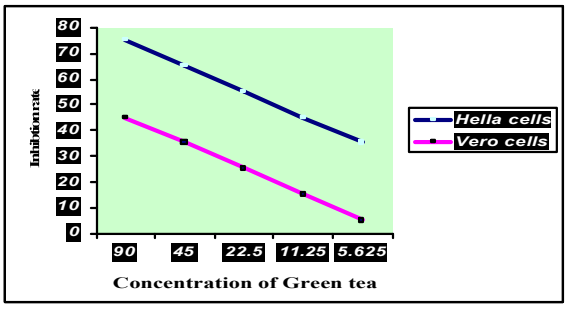


Fig. 3: Anti-cancer effect of Green tea aqueous extract on Hella and Vero cell lines

Anticancer activity

In vivo: Post mortem examination of sacrificed rates given DEC (group 1), revealed enlarged, hyperplastic livers with some adhesions and hepatocellular adenoma with sever and diffused granular degeneration and cell swelling in all rats of this group. Rats in other groups showed apparently normal livers with healthy cells (Microphotograph 1).

In vitro: Aqueous extracts of Ns and GT revealed *in vitro* anticancer activities, whereas, both inhibited the rate of tumor cell proliferation and viability at different concentrations on Hella and Vero cell lines (Fig. 2 and 3). Ns showed inhibition rate of 80 and 65% at the highest dosage (80µM), while GT showed inhibition rate of 75 and 45% at the highest dose (90µM) for Hella and Vero cells, respectively.

Anti-angiogenic activity: Noticeable increases in endothelial multiplication of aorta sections in wells containing both NS and GT were obvious (Microphotograph 2).

Antioxidant activity

DPPH radical scavenging activity: The two plant extracts (Ns and GT) exhibited a significant dose dependant inhibition of DPPH activity, with a 50% inhibition (IC_{50}) at concentrations of 2 and 3µg/ml for GT and Ns, respectively (Fig. 4).

Nitric oxide radical inhibition activity: The scavenging of nitric oxide by plant extracts was increased in a dose-dependant manner as illustrated in Fig. 5. 50% of NO generated by incubation was scavenged at concentrations of 116 and 110µg/ml for GT and Ns extracts, respectively.

Lipid peroxidation activity: Activity of both plant extracts against non-enzymatic lipid peroxidation in rat liver microsomes is shown in Fig. 6. Addition of Fe^{2+}/ ascorbate to the liver microsomes cause an increase in lipid peroxidation. The extract showed inhibition of peroxidation in all concentrations with 50% inhibition effect at 104 and 108µg/ml for GT and Ns, respectively.

Microphotograph 2: Anti-angiogenic activity of Ns and GT. A, negative control (only maintenance medium; MM) with normal formation of new small capillaries. B and C, cross sectioned aorta supplemented with Ns and MM (B) or MM and GT (C) with decreased endothelial cell multiplication and angiogenesis (X=100)

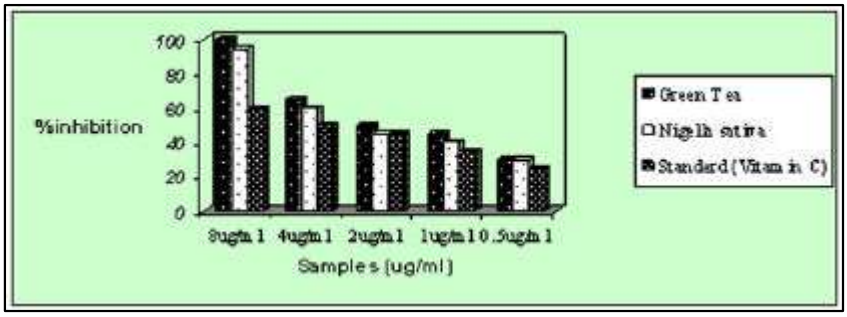


Fig. 4: Scavenging effect of NS and GT extracts on DPPH radical using vitamin C as a standard

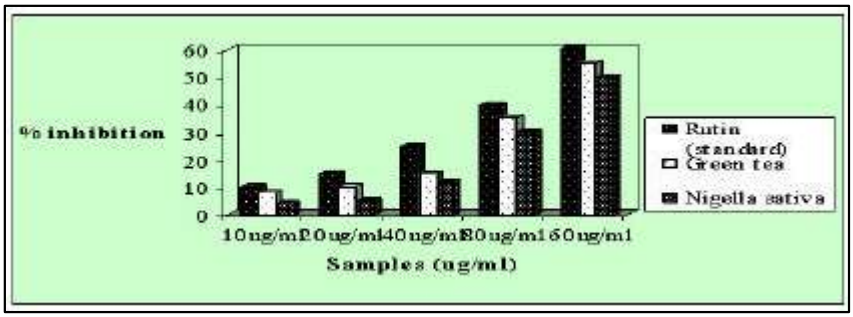


Fig. 5: Scavenging effect of Ns and GT extracts using rutin on nitric oxide radical as a standard

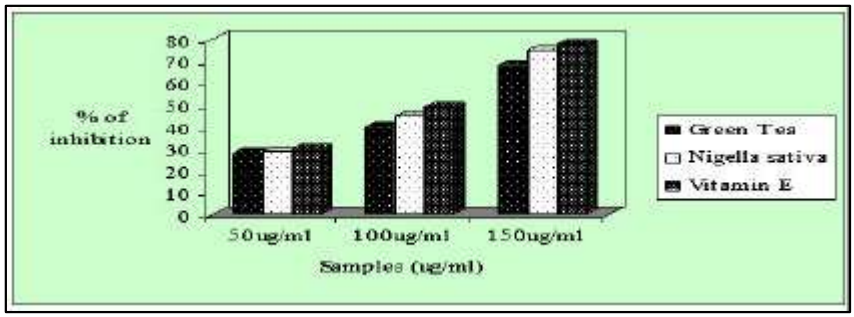


Fig. 6: Effect of Ns and GT extracts on lipid peroxidation of liver microsome induced by Fe2+/ascorbate using vitamin E as a standard

DISCUSSION

The current work was designed to evaluate the possible biological effects of some commonly used plants; *Nigella sativa* and green tea, since these plants are commonly used as remedies long time ago.

Investigation herein revealed that Ns and GT have anti-viral, cancer, angiogenic and oxidant activities. The obvious anti viral activity of Ns coincides with the result of [1, 2, 26] who found that NS contains materials known as Nigellon, thymoquinon and thymohydroquinone that possess antimicrobial effect and enhance the production of interlukin-3 and 1ß which has an effect on microphages. In the same time [5], reported that the main active antiviral components of GT are Epigallocatechin-3-Gallate (EGCG), Epicatechin Gallate (ECG) and Epigallocatechin (EGC) which have potential direct virucidal effect as well as an indirect effect on viruses by increasing the levels of INF-? [3], however, GT give more satisfactory results than Ns (EC_{50} of 35and 4.22 µM, respectively).

From the anti-carcinogenic point of view, a recent short-term study by [4] has shown that DEN-induced expression of the p-isoform of glutathione S-transferase (GST-P) in rat livers could be significantly inhibited by treatment of rats with Ns and other plants and that come in agreement with the current result. In the same time, one of the most notable features in livers of rats treated only with DEN, was the extensive inflammation which was completely absent in livers of rats treated with DEN+ NS and DEN+GT and this was explained by recent investigations which have shown that the effects of these natural products have related to their ability to reduce inflammation and/or vascular permeability or production of detrimental eicosanoids and other angiogentic factors [27]. This finding also met with the results of this study as anti-cancer and anti-angiogenic. Also, both extracts gave better results when applied on Hella than that given when applied on Vero cell line. GT gave better results when applied on Hella cells than Ns, while Ns gave butter results on Vero cells than that given by GT.

As an antioxidant, DPPH is a free radical, stable at room temperature, which produces a purple colour solution in methanol. It is reduced in the presence of an antioxidant molecule, giving rise to uncolored methanol solution. However, the results of this study come in agreement with the reported value of [28]. GT gave the best results, while Ns gave better results than vitamin C. Also, NO radical inhibition study proved that aerial part of the extract is a potent scavenger of NO. This NO generated from sodium nitro preside reacts with oxygen to form nitrite. The extract inhibits nitrite formation by competing with oxygen to react with NO directly and also, to inhibit its synthesis. Scavengers of NO compete with oxygen leading to reduced production of NO [29]. However, rutin gave the best results followed by GT then Ns. The liver microsomal fraction undergoes rapid non-enzymatic peroxidation when incubated with $FeCl_3$ and Ascorbic acid. The use of Fe (III) in the presence of a reducing agent such as ascorbate produces -OH and they attack the biological material. This leads to the formation of Malonodialdehyde (MDA) and other aldehydes, which form a pink chromogen withTBA [30]. The best results were achieved by GT followed by Ns then vitamin E.

In conclusion, results of the current investigation clearly proved the efficacy of using *Nigilla sativa* and Green tea in traditional medicine. However, further studies must be performed to detect the proper dosage before field application.

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