Anti-Cancerous Effect of
*Nostoc sphaeroides*
7.1. INTRODUCTION

Cancer is a genetic disease because it can be traced to alterations within specific genes, but in most cases, it is not an inherited disease. In an inherited disease, the genetic defect is present in the chromosomes of a parent and is transmitted to the zygote. In contrast, the genetic alternations that lead to most of the cancers arise in the DNA of a somatic cell during the lifetime of the affected individual. Because of these genetic changes, cancer cells proliferate uncontrollably, producing malignant tumors that invade surrounding healthy tissue. As long as the growth of the tumor remains localized, the disease can usually be treated and cured by surgical removal of the tumor. But malignant tumors tend to metastasize, that is, to form a spawn cell that make away from the parent mass, enter the lymphatic or vascular circulation, and spread to distant sites in the body where they establish lethal secondary tumors that are no longer amenable to surgical removal (Ishizaki et al., 1995).

A genetic basis for human carcinogenesis has been established through biochemical and molecular analyses of the disease. Many different types of human cancers have been caused by occupational exposure while others have been attributed to environmental exposure to chemical and/or viral agents. Chemical carcinogenesis is a multistage process that begins with exposure usually to complex mixtures of chemicals that are found in the human environment. Carcinogenesis can be divided conceptually into 4 steps, tumor initiation, tumor promotion, malignant conversion and tumor progression. Tumor initiation results from irreversible genetic damage. A chemical carcinogen causes a mutation by modification of the molecular structure of the DNA. Tumor promotion comprises the selective clonal expansion of initiated cells. Clonal expansion of initiated cells produces a
larger population of cells that are at risk of further genetic changes and malignant conversion. Tumor promoters generally are nonmutagenic, are not carcinogenic alone and often are able to mediate their biologic effects without metabolic activation. There are certain chemicals capable of both tumor initiation and promotion. They are called complete carcinogens. Examples are benzo (a) pyrene and 4 aminobiphenyl. Malignant conversion is the transformation of a preneoplastic cell into one that expresses the malignant phenotypes by further genetic changes. Tumor progression comprises the expression of the malignant phenotype and the tendency of already malignant cells to acquire more aggressive characteristics in time.

Because of its impact on human health and the hope that a cure might be developed, cancer has been the focus of a massive research effort for decades. Though these studies have lead to a remarkable breakthrough in our understanding of the cellular and molecular basis of cancer, they have had very little impact on their preventing the occurrence of or increasing the chances of surviving most cancers. Current treatments, such as chemotherapy and radiation, lack the specificity needed to kill cancer cells without simultaneously damaging normal cells, as evidenced by the serious side effects that accompany these treatments. As a result, patients cannot usually be subjected to high enough doses of chemicals or radiation to kill all of the tumor cells in their body. Cancer researchers have been working for many years to develop more effective and less debilitating treatments.

The role of polycyclic aromatic hydrocarbons (PAH) are clearly implicated in the process of carcinogenesis especially 7, 12-dimethylbenz[a]anthracene (DMBA) which is one of the most potent skin carcinogens known. Most of the metabolically activated PAHs are
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mutagenic to DNA. 12-O-tetradecanoylphorbol-13-acetate (TPA) is a skin tumor promoter isolated from seed oil of *Croton tiglium* and has been extensively studied in DMBA induced mouse skin tumor model. Inflammation and free radicals have been associated with cancer in various tissues including skin, bladder, stomach and colon. The experimental evidences strongly suggested the role of free radical mediated tumor promotion in phorbol ester promoted papilloma on mouse skin (Lewis and Adams, 1987). The application of croton oil has been shown to reduce antioxidant enzymes in both epidermal and inflammatory cells (Solanki *et al*., 1981). Inhibition of ROI generation can serve as an important system for the identification of the agents that inhibit oxidative damage to DNA as well as tumor promotion.

Chemotherapy is an effective treatment against cancers either singly or in combination with surgery and/or radiotherapy. In chemotherapy, drugs like cisplatin, carboplatin, cyclophosphamide, doxorubicin, melphalan, mictomycin, gemcitabine etc have been used for the treatment of cancers. However therapeutic efficiency of most of them are limited due to the development of various side effects in the host and/or the acquired drug resistance by the cancer cells (Black and Livingston, 1990). In an attempt to abate these side effects and better remedy against various malignancies, many plant derivatives have been used with varying success. Certain species of cyanobacteria have been found to possess significant pharmacological activities and physiological properties such as prevention and improvement of conditions in cancer and heart diseases. Some species of cyanobacteria have been found to markedly inhibit the growth of different kinds of tumors (Mathew *et al*., 1995). Cyanobacteria and the study of their medicinal value has become a subject of great interest.
Extracts of the *N.sphaeroides* were evaluated for their *in vivo* and *in vitro* inhibition of tumor promoting activity was determined using the classic two-stage carcinogenesis model in mouse skin.

**7.2. MATERIALS AND METHODS**

**7.2.1. Preparation of the extracts of *N. sphaeroides***

Ethanol extract of *N. sphaeroides* was prepared as described in the section 3.2.1.

**7.2.2. Animals**

Female Balb/c mice (25 ± 2 g) were used for the study.

**7.2.3. Determination of anti-promotional activity using two-stage carcinogenesis**

Female Balb/c mice were shaved on their back using surgical clippers 2 days before the experiment. Animals with complete hair growth arrest were grouped into 3 groups of 10 animals each. The skin tumor was initiated with a single topical application of 390 nmol of 7, 12-dimethylbenz[a]anthracene (DMBA) in 200 µl acetone. One week after tumor initiation, the promotion was induced by topical application of 200µl of croton oil (10% in acetone, v/v) twice weekly for 8 weeks to the same area (Verma and Boutwell, 1980). The ethanol extract of *N. sphaeroides* (1mg and 5mg in 200 µl acetone/mouse) was applied topically 40 minutes before each croton oil application. The group treated with croton oil alone served as positive control. Skin papilloma formation was recorded weekly in each experimental group. Average number of papilloma per mouse, and tumor latency period were recorded.

**7.2.4. Determination of cytotoxicity screening and apoptogenic activity of *N.sphaeroides***

The cytotoxicity screening of ethanol extract of *N. sphaeroides* was determined by using MTT assay (section 3.2.34) and the apoptogenic
potential was determined by using mitosensor assay (section 3.2.35), DAPI assay (section 3.2.36) and caspase assay (section 3.2.37).

7.3. RESULTS

7.3.1. Cytotoxicity screening with ethanolic extract of N. sphaeroides by MTT assay.

A panel of human cell lines consisting of MCF 7, SW 480, HeLa, HCT 116 and IMR 32 were seeded in microtitre plates (5000 cells/well) and allowed to grow until 85% confluence was reached. Then the medium was removed and ethanolic extracts of *N. sphaeroides* were mixed with medium in four different concentrations (37.5, 75, 150 and 300µg/ml) were added. The cells were seeded in duplicates and one plate was assayed after 24 hours of incubation and the other plate was assayed after 48 hours of incubation by MTT assay. For control cells, medium without drug was added. The results of the MTT assay for different cell lines are the following.

The Fig 7.1 reveals the result of the MTT assay for MCF 7 cell line. There was an increase in percentage of cytotoxicity in a dose and time dependent manner. Fifty-percentage cell death was observed for the drug concentration of 150µg for 24 and 48 hours of incubation.

The results of MTT assay for SW 480 cell line is plotted in Fig 7.2. The percentage of cell death increases in a time and concentration dependent manner on MTT assay. Fifty percentage cell death was observed for the drug concentrations of 300µg and 150µg for 24 and 48 hours of incubation respectively.

The results of MTT assay for HeLa cell line is depicted in Fig 7.3. The percentage of cytotoxicity was increased in a time and dose dependent manner. Fifty percentage cell death was observed for the drug
concentrations 300µg and 150µg for 24 and 48 hours of incubation respectively.

The Fig 7.4 reveal the result of the MTT assay for HCT 116 cell line. There was an increase in percentage of cytotoxicity in a dose and time dependent manner. Fifty percentage of cell death was observed for the drug concentrations of 150µg and 75µg for 24 and 48 hours of incubations respectively.

The results of MTT assay for IMR 32 cell line is plotted in Fig 7.5. The percentage of cell death increases in a time and concentration dependent manner on MTT assay. Fifty percentage cell death was observed for the drug concentration 150µg for 24 and 48 hours of incubation.

7.3.2. **Determination of anti-promotional activity of ethanol extract of *N. sphaeroides***

Topical application of ethanol extract inhibited skin papilloma initiated by DMBA and promoted by croton oil on mouse skin (Fig 7.6). Group of animals applied with croton oil and DMBA showed 85% tumor incidence at 15 weeks after DMBA treatment. Application of ethanol extract of *N. sphaeroides* prior to croton oil application reduced the percent of incidence. Topical application of extract at a dose of 1mg showed 60.1% incidence at 15 weeks and at a dose of 5 mg showed 39.6% incidence at 15 weeks as compared to control (85% incidence) (Fig 7.7). The percentage of animals with tumor in the control group attained maximum at 7 weeks after tumor promotion by croton oil. The average number of tumor (1mm diameter) per animal in the control group was six numbers 7 weeks after the croton oil application, while the average number of tumor per animal in the 1 and 5mg extract treated
groups of animals were two and one respectively (Fig 7.8) and (Plate 7.1). The tumor latency period in the control, extract 1 and 5 mg treated groups were 38, 45 and 56 days respectively (Fig 7.9). The time for first tumor induction in control, EEN 1mg and EEN 5mg treated groups were 7, 9 and 12 weeks respectively. The body weight of mice bearing papilloma was found to be increased as compared to mice treated with EEN. The body weight in control, EEN 1mg and EEN 5mg treated groups were 1.5g, 2.8g and 3.4g respectively (Fig 7.10).

**Figure 7.1: Percentage toxicity for MCF 7**

![Graph of cancer cell death for MCF 7](image)

**Figure 7.2: Percentage toxicity for SW 480**

![Graph of cancer cell death for SW 480](image)
Figure 7.3: Percentage toxicity for HeLa

Figure 7.4: Percentage toxicity for HCT 116

Figure 7.5: Percentage toxicity for IMR 32
Plate 7.1: Effect of ethanol extract of *N. sphaeroides* on DMBA induced and croton oil promoted skin papilloma on mice skin.

(a) Control (DMBA + croton oil)
(b) DMBA + croton oil + EEN (1mg/kg body wt.)
(c) DMBA + croton oil + EEN (5mg/kg body wt.)
Figure 7.6: Effect of EEN on croton oil induced tumor promotion in mouse skin.

Figure 7.7: Time for first tumor induction

Figure 7.8: Percentage of tumors at 15th week
Figure 7.9: Average number of tumor per animal

Figure 7.10: Increasing in body weight of mice bearing papilloma (in grams).
7.3.3. Apoptogenic activity of Nostoc sphaeroides

Apoptogenic potential of EEN was estimated by observing nuclear condensation or pyknosis of EEN treated cell lines change in the mitochondrial membrane potential and cytochrome c release of EEN treated cell lines. The induction of caspases in the EEN treated cell lines were also estimated to find out the induction of apoptosis.

Chromatin condensation is a clear indication of apoptosis and this nuclear condensation can be visualised by staining the nuclei by a nuclear dye such as DAPI (4,6 diamidineo-2 phenylindole). The results of DAPI assay for the cell lines MCF 7, SW 480, HeLa, HCT 116 and IMR 32 were shown in Figure 7.11 and Plate 7.2 to 7.6 respectively. In MCF 7, HCT 116, HeLa and IMR 32 cell lines treated with EEN, clear chromatin condensation was observed. When these cell lines were treated with EEN at a concentration 150 µg/ml showed 50 % nuclear condensation after 24 hours. The SW 480 and HeLa cell line showed clear chromatin condensation at 300 µg/ml EEN for 24 hours. SW 480 colon cancer cell line is there fore less sensitive to EEN when compared to others. But in untreated control cells, all the five cell lines showed a homogenous nuclear chromatin indicative of healthy growth.
Plate 7.2  Nuclear condensation in MCF 7 cells
(a) Control (b) 37.5 µg EEN (c) 75 µg EEN (d) 150 µg EEN  
(e) 300 µg EEN
Plate 7.3  Nuclear condensation in SW 480 cells

(a) Control  (b) 37.5 µg EEN  (c) 75 µg EEN  (d) 150 µg EEN
(e) 300 µg EEN
Plate 7.4  Nuclear condensation in HeLa cells
(a) Control (b) 37.5 µg EEN (c) 75 µg EEN (d) 150 µg EEN
(e) 300 µg EEN
Plate 7.5  Nuclear condensation in HCT 116 cells
(a) Control (b) 37.5 μg EEN (c) 75 μg EEN (d) 150 μg EEN
(e) 300 μg EEN
Plate 7.6  Nuclear condensation in IMR 32 cells

(a) Control (b) 37.5μg EEN (c) 75 μg EEN (d) 150 μg EEN
(e) 300 μg EEN
Mitochondria play a key role in the apoptotic machinery of the cell by releasing caspase activators such as Cytochrome c, releasing caspase independent death effectors and causing the loss of essential mitochondrial functions (Kroemer et al., 1997). During apoptosis, the outer mitochondrial membrane becomes permeabilized, allowing intermembrane proteins to be released and activate the downstream apoptotic machinery (Marchetti et al., 1997). The mitochondrial membrane permeability of treated cell was visualized by staining with JC-1 dye. Dye gives a red fluorescence when the mitochondrial membrane is intact and gives a green fluorescence when the membrane is damaged as in the case of apoptosis. The Plates 7.7, 7.8, 7.9, 7.10 and 7.11 show the results of mitosensor assay in cell lines MCF 7, SW 480, HeLa, HCT 116 and IMR 32 respectively in all the 5 cell lines. The plates clearly indicate that EEN induced change in mitochondrial membrane potential in a dose dependent manner. In cells treated with EEN, the intensity of green fluorescence is more as compared to the untreated control where red fluorescent patches of mitochondria were observed.
Plate 7.7: Mitochondrial membrane permeabilisation in MCF cells
(a) Control (b) 37.5 µg EEN (c) 75 µg EEN (d) 150 µg EEN
(e) 300 µg EEN
Plate 7.8: Mitochondrial membrane permeabilisation in SW 480 cells
(a) Control (b) 37.5 µg EEN (c) 75 µg EEN (d) 150 µg EEN
(e) 300 µg EEN
Plate 7.9: Mitochondrial membrane permeabilisation in HeLa cells
(a) Control (b) 37.5 µg EEN (c) 75 µg EEN (d) 150 µg EEN
(e) 300 µg EEN
Plate 7.10: Mitochondrial membrane permeabilisation in HCT 116 cells
(a) Control (b) 37.5 μg EEN (c) 75 μg EEN (d) 150 μg EEN (e) 300 μg EEN
Plate 7.11: Mitochondrial membrane permeabilisation in IMR 32 cells
(a) Control  (b) 37.5 µg EEN  (c) 75 µg EEN  (d) 150 µg EEN  (e) 300 µg EEN
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The intracellular transmission of apoptotic stimulus is regulated by a well organized system of initiator and effector caspases. Induction of caspases is a late apoptotic event, which shows that cells are committed to apoptosis. In MCF 7, cell lines treated with EEN, 24 hours at different concentrations namely 37.5, 75, 150 and 300µg/ml, there was an increase in caspase activity in a dose dependent manner as shown in Figure 7.12. The cell treated with 37.5 and 75 µg/ml of EEN, there was 11.56% and 24.69% increase respectively in total caspase activity as compared to control. In cells treated with 150 µg/ml and 300 µg/ml EEN there was an increase of 56.31% and 60.36% respectively as compared to control cells.

Fig. 7.12. Percentage increase in total caspase activity in MCF 7 cells treated with EEN
7.4. DISCUSSION

In the present study the *in vitro* anti-proliferative potential of the EEN was determined and the results are discussed. Therefore, an attempt was made to find out, whether the anti-proliferative action was due to apoptogenic effect of the EEN. For this three different experiments were conducted. Chromatin condensation and apoptotic body formation was confirmed by morphological evaluation of the EEN treated cells by DAPI staining. In MCF 7 cells treated with EEN, there was an increase in the cells showing apoptotic morphology in a dose dependent manner. Apoptosis typically affects cells that are aged, dysfunctional, or damaged by external stimuli. It is an active, energy requiring process leading to a well regulated degradation of the cell. Early pathomorphological features are chromatin condensation marginalisation in the nucleus, DNA fragmentation into mono and oligo nucleosomal units, cellular shrinkage, packing of organelles and dilatation of the endoplasmic reticulum (Holdenrider and Stieber, 2004). Quantifying cell death and cellular proliferation can provide information about the process of carcinogenesis and the response to anti-tumor treatment. Since the EEN could induce apoptosis in cancer cell lines as evidenced by DAPI staining and morphological observation of condensation, it can be said that the EEN has promising anti-cancer potential. Hengartner, (2000) reported that depending on type and dosage of the chemotherapeutic drugs, the modality of radio therapy and the sensitivity of tissue, cellular damage, mostly results in arrest of the cell cycle and in response to insufficient repair leads to induction of active apoptotic cell death.

In the second experiments, all of the five different cell lines were assayed for the loss of mitochondrial membrane potential by JC-1 dye.
This is a cationic fluorescent dye, staining the mitochondria. In the healthy cells, the dye stains the mitochondria bright red. The negative charge established by the intact mitochondrial membrane potential allows the lipophilic dye bearing a delocalized positive charge, to enter the mitochondrial matrix where its accumulates (Smiley et al., 1991). When the critical concentration is exceeded, JC-1 aggregates from which become fluorescent red. In apoptotic cells the mitochondrial membrane potential collapses and the JC-1 cannot accumulate within the mitochondria. In these cells JC-1 remains in the cytoplasm in a green fluorescent monomeric form. Apoptotic cells showed primarily green fluorescence are easily differentiated from healthy cells, which show red and green fluorescence. The mitochondrial permeability transition was an important step in the induction of cellular apoptosis. During apoptosis the electrochemical gradient referred to as ΔΨ across the mitochondrial membrane collapses. The collapse is thought to occur through the formation of pores in mitochondria by dimerised Bax or activated Bid, Bak and Bad proteins. Activation of the pre-apoptotic proteins is accompanied by the release of cytochrome c into the cytoplasm. (Luo et al., 1998). From the Figure 7.7 to 7.11, it is evident that EEN could induce cytochrome c release by damaging the mitochondrial membrane. In all the cell lines treated with EEN there was remarkable release of cytochrome c into the cytosol in a dose dependent manner, compared to the controls. This supports the claim that EEN could induce apoptosis. The release of cytochrome c into cytosol is caused by pores created by pro-apoptotic proteins (Desagher et al., 1999). So it is evident that pro-apoptotic proteins of intrinsic pathway of apoptosis are induced by the treatment with EEN. Mitochondrial play a key role in the apoptotic machinery of the cell by releasing caspase activators such as cytochrome
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c, releasing caspase independent death effectors and causing the loss of essential mitochondrial functions (Green and Kroemer, 2004). During apoptosis, the outer mitochondrial membrane becomes permeabilised, allowing intermembrane proteins to be released and activate the downstream apoptotic machinery, including cytochrome c-procaspase 9 complex and activation of caspase 9 (Orrenius, 2004). Mitochondrial membrane permeabilisation (MMP) alone has been found to be a sufficient trigger of apoptosis or necrosis, even in cancer cells (McLaehlan et al., 2005). If the EEN is able to overcome the cancer cells resistance to EEN, then the cells will have no choice but to undergo apoptosis. Induction of apoptosis is an effective mechanism used to eradicate transformed or deleterious cells. Many chemotherapeutic or chemopreventive agents act through triggering of apoptotic pathways in tumor cells (Kutuk et al., 2005). The cellular apoptotic machinery is formed by protein interactions and protein modification. Protein kinases as well as various cystein–specific aspartate proteases or caspases have been proposed to medicate apoptosis induced by cytokines, chemotherapeutics and cellular stress through a highly organized network at different signaling level (Daniel et al., 2000).

In the third experiment the cell death associated caspases (caspases 2, 3,7,8,9 and 10) were collectively assayed by using the common caspase substrate. As shown in figure 7.9, the EEN dose of 150µg/ml showed a 50% increase in caspase activity in treated cells as compared to control cells. The increased caspase activity in the EEN treated cell compared to control cells supported that the treated cells were undergoing apoptotic cell death. From the above mentioned studies showing DNA damage, MMP and caspase activation it is evident that EEN is able to induce
apoptosis in cancer cell lines. So the EEN mechanism by which the EEN effects cell death in cancer cells and prevents its proliferation might be apoptosis. The evidences indicate that the metabolic activation of DMBA occurs primarily through the formation of a 3,4-diol-1,2-epoxide. ROS production by double or multiple TPA treatments is closely associated with the metabolic activation of proximate carcinogens and increased level of oxidized DNA bases. Persistent oxidative stress in cancer may also constantly activate transcription factors, such as NF-kB, through the intracellular signal transduction system and induce expression of protooncogenes such as c-fos, c-jun and c-myc (Toyokumi et al., 1995). Oxidative stress induces DNA damage such as modified base products and strand breaks that may lead to further mutation and chromosomal aberration, in the single mutated clones.

Experimental results indicate that application of ethanol extract of *N. sphaeroides* before each application of croton oil directly scavenge the free radical or inhibited the generation of free radicals. This was evident from decreased skin lipid peroxidation induced by croton oil when pre-treated with the extract (Section 5.3.3). The extract also inhibited the croton oil mediated skin inflammation. Hence the anti-promotional activity of the extract was probably mediated through the radical scavenging activity of the extract. The tumor latency period in animals that were treated with 5mg was extended significantly compared to the control as well as 1mg treated group. The average number of tumor/animal is also decreased in the extract treated groups compared to the control group. The results indicate the efficiency of the extract in delaying the skin tumor incidence in animals. C-Phycocyanin (C-PC) from blue-green algae has been reported to have various pharmacological
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characteristics, including anti-inflammatory and anti-tumor activities (Wang et al., 2006).

C-phycocyanin (C-PC), a water-soluble protein pigment, isolated from Spirulina platensis, is of great importance because of its various medical and pharmacological properties. In the present study, we first investigated the effect of highly purified C-PC on growth and proliferation of HeLa cells in vitro. The results indicated that there was a significant decrease in the number of cells that survived for HeLa cells treated with C-PC compared with control cells untreated with C-PC. Further electron-microscopic studies revealed that C-PC could induce characteristic apoptotic features, including cell shrinkage, membrane blebbing, microvilli loss, chromatin margination and condensation into dense granules or blocks (Li, et al., 2006). The effect of highly purified C-PC was tested on growth and multiplication of human chronic myeloid leukemia cell line (K562). The results indicate significant decrease (49%) in the proliferation of K562 cells treated with 50 µM C-PC up to 48 h. Further studies involving fluorescence and electron microscope revealed characteristic apoptotic features like cell shrinkage, membrane blebbing and nuclear condensation (Subhashini et al., 2004).

Lophocladine B (2) an alkaloid isolated from marine red algae Lophocladia species exhibited cytotoxicity to NCI-H460 human lung tumor and MDA-MB-435 breast cancer cell lines (Gross et al., 2006). It has been reported that Bromophycolides C-I (1-7) were isolated from extracts of the Fijian red alga Callophycus serratus exhibited anti-neoplastic activity against a range of human tumor cell lines (Kubanek et al., 2006). The crude lipid extract of Indonesian red alga Vidalia species was showed anticancer property (Yoo et al., 2002). Dietary Laminaria and Porphyra species have
been reported to reduce the risk of intestinal or mammary cancer in animal studies. Algal anti-carcinogenicity may involve effects on cell proliferation and antioxidant activity. It was established that the extracts of red alga, dulse (*Palmaria palmata*) and three kelp (*Laminaria setchellii, Macrocystis integrifolia, Nereocystis leutkeana*) showed significant decrease in the proliferation of human cervical adenocarcinoma (Yuan and Walsh, 2006). It has been reported that seven brominated diterpenes of the parguerene and isoparguerene series were isolated from the red alga *Jania Rubens* (L.) Lamx, collected from the Red Sea coast at Hurghada, Egypt. The diterpenes were identified as isoparguerol, isoparguerol-16-acetate, isoparguerol-7, 16-diacetate, parquerol-16-acetate, parquerol-7, 16-diacetate, deoxyparguerol and deoxyparguerol-7-acetate. The isolated diterpenes had a marked anti-tumor activity (Award, 2004). From the above results it can be concluded that EEN has effective anti-tumor activity.