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Potent Anticancer and Antioxidant Activities of Active Ingredients Separated from *Solanum nigrum* and *Cassia italica* Extracts

Ahmed M. ABOUL-ENEIN*[1)], Faten Abu EL-ELA[1)], Emad SHALABY[1)] and Hany EL-SHEMY[1)]

Abstract: The present work discusses the separation and purification of bioactive compounds from the crude ethanolic extracts of *Cassia italica* and *Solanum nigrum* leaves. Identification of compounds in the crude extract was accomplished using different spectrophotometric analyses (MS, NMR and IR). Eight active compounds were identified from crude extract of *S. nigrum* while six were identified from *Cassia italica*. Each active compound was further evaluated for anticancer properties using Ehrlich ascites carcinoma cell (EACC) line and Hepatoma cell (HepG2) line. The antioxidant activity of each active compound was determined using the 2, 2 diphenyl dipicryl hydrazine (DPPH) method. The identified compounds showed variable antioxidant activities. It is apparent from our study that effective drugs produced from *Solanum nigrum* and *Cassia italica* tend to support the use of these plants in the treatment of cancer.

Key Words: Apoptosis, Biological activities, Cassis italic, Pure compounds, *Solanum nigrum*

1. Introduction

Medicinal plants have been used by humans for centuries in folklore medicine (Sermakkani and Thangapandian, 2012). Medicinal plants are also incorporated into the historical medicine of virtually all human cultures. The plants are a rich source of secondary metabolites with interesting biological activities. Therefore, these secondary metabolites have an important source with a variety of structural arrangements and properties (Vickers, 2002; El-Shemy *et al*., 2003, 2007).

Solanum nigrum (black nightshade) is known as a natural medicinal plant. *Solanaceae* is well known and has been screened by researchers for their medical actions. The plants were extensively used to treat severe diseases such as pain, inflammation and fever (Acharya and Pokhrel, 2006). As well, plants were used as an antitumorigenic, antioxidant, anti-inflammatory, hepatoprotective, diuretic, and antipyretic agent (Aboul-Enein *et al*., 2012; Zakaria *et al*., 2006). As stated by Desphande and Bhalsing (2013): "an extensive survey of literature revealed that *Cassia* is an important source of many pharmacologically and medicinally important chemicals (Skehan *et al*., 1990), several studies have claimed the use of some species of *Cassia* for the treatment of various diseases but still the pharmacological potential of the other plants species of the genus are required to be explored (Mazumder *et al*., 2009; Riboli and Norat, 2003)."

This work aimed to study the influence of isolated and identified pure compounds from ethanolic extracts of *Cassia italica* and *Solanum nigrum* leaves which showed antioxidant and anticancer agents.

2. Materials and Methods

2.1. Collection of plant materials

Wild growing *Solanum nigrum* and *Cassia italica* were collected during February - March, 2012 from El-Alaemeen -Coastel area, Sedi Abd El-Rahman, north-west of Egypt. Whole plant samples were air dried and leaves were ground to a powder using a mechanical mortar.

2.2. Preparation of extracts

Ethanolic extracts of *S. nigrum* and *Cassia italica* were prepared according to Ferrigni *et al*. (1982). Fifty grams of ground leaves were extracted with 70% ethanol. The supernatant was separated and the solvent was removed. All the extracted materials were used to test for antioxidant and anticancer activities. The remainder of the plant materials was preserved at -20°C for spectrophotometric analysis.

2.3. Anticancer activity (Cytotoxicity)

2.3.1. Viability of Ehrlich Ascites Carcinoma cells (EACC)

A line of Ehrlich Ascites Carcinoma from National Cancer Institute (NCI) Cairo, Egypt was used. The viability percentages of tumor cells (2×10^4 cells) after incubation for 2 h with ethanolic extract (100 μg/ml) as well as with saline as control were measured by the modified cytotoxic trypan blue-exclusion technique of Bennett *et al*. (1976). For all

* Corresponding Author: aboul_enein1@yahoo.com
Giza, Egypt
1) Biochemistry Department, Faculty of Agriculture, Cairo University

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examined materials (and control), 10 µl of cell suspension was mixed with 80 µl saline and 10 µl trypan blue (0.4%) then the number of living cells (non-stained) was calculated using a homocytometer slide by microscope.

2.3.2. HepG2 cell line

The anticancer activity of the crude extract and the fractionated compounds were tested against HepG-2 cells (Vacsera, Egypt) by SRB assay as previously described (Skehan *et al*., 1990) or by neutral red assay (Retpetto *et al*., 2008). Exponentially growing cells were collected using 0.25% Trypsin-EDTA and plated in 96-well plates at 1000-2000 cells/well. Cells were exposed to each test compound for 72 h and subsequently fixed with TCA (10%) for 1 h at 4ºC. After several washings, cells were exposed to 0.4% SRB solution for 10 min in the dark and subsequently washed with 1% glacial acetic acid. After drying overnight, Tris-HCl was used to dissolve the SRB-stained cells and color intensity was measured at 540 nm.

2.3.3. Caspase activity assay

After centrifugation of the treated cells as previously described, caspase-3 enzymatic activity was determined using CasPASE Apoptosis Assay Kit (Geno Technology Inc. St. Louis MO, USA). Prior to use, caspase kit reagents were first prepared, followed by lysis of the treated cells (HepG2) according to a modification of the manufacturer's protocol. In this study, cells were lysed with a sonicator and caspase-3 enzymatic activity in the lysates was determined as described. It essential to mention that the level of caspase-3 (CPP32) enzymatic activity in the cell lysate was directly proportional to the color reaction read at 405 nm. The inter-treatment data were compared to ascertain and confirm the effect of ZVAD-FMK on caspase-3 enzymatic expression.

2.4. Antioxidant activity

The DPPH (2,2-diphenyl-1-picrylhydrazyl) test was carried out as described by Breitenbucher and Figliozz (2000). One ml of plant extract (100 µg/ml) was mixed with 1ml DPPH reagent {0.002% (w/v)/ methanol solution}. After incubation for 30 min in the dark at room temperature, the absorbance was measured at 517 nm.

2.5. Identification of the active compounds

The crude ethanolic extracts of *C. italica* and *S. nigrum* were first fractionated using TLC technique to separate the different fractions using different combinations of petroleum ether/ethyl acetate solvents (9:1, 8.5:1.5, 8:2, 7:3 and 5:5) as the mobile phase. The separated fractions (1-10 which have best effect) were scratched and eluted with the same mobile phase, filtered, evaporated, weighed and then used in bioassays. The pure fractions were analyzed by different spectrophotometric techniques.

2.5.1. Mass spectroscopic (MS) analysis of potent fractions

The potent fractions of *Cassia italica* and *Solanum nigrum* were analyzed by Mass spectrum (MS). The mass spectrometer was scanned over the 40 to 500 m/z range with an ionizing voltage of 70 eV and identification was based on standard mass library of National Institute of Standards and Technology (NIST Version 2.0) to detect the possible fraction structure.

2.5.2. Fourier transformed infra red (FTIR) spectra

A Perkin Elmer (Waltham, Massachusetts, USA) was used to obtain Fourier transformed infrared (FTIR) spectra (System 2000) and applied for each pure fraction analysis.

2.5.3. Proton magnetic resonance spectra (^{1}H NMR)

The identification of compounds was confirmed by carrying out 1H-NMR analysis using NMR Joel GIM, EX 270 (400 Hz).

2.5.4. Statistical analysis

Data were subjected to an analysis of variance, and the means were compared using the "Least Significant Difference (LSD)" test at 0.01 levels, as recommended by Snedecor and Cochran (1982). Data are presented as mean ± SD.

3. Results and Discussion

The examined wild plants were used for their biological evaluation and the obtained results were illustrated and discussed.

3.1. Antioxidant activity

The change in absorbance produced by reduced DPPH was used to evaluate the ability of tested compounds as antioxidant activity. As shown in **Table 1** (for *S. nigrum* extract), band (2) came in the first rank which gave the highest antioxidant activity (80.5%), **Figure 1**. The two bands (3, 4) came in the second category which gave antioxidant activity as 65.6 and 50.5%, respectively, while the other five bands gave antioxidant activity less than 50%. The antioxidant activity of ten bands isolated from of *Cassia italica* was evaluated and results showed that two isolated bands (3 and 5) gave antioxidant activity more than 60% while the four bands (1, 2, 4 and 6) gave activity less than 60% (45.8, 48.7, 49.5 and 56.7, respectively). The other bands gave less than 40% antioxidant activity. The fractions which have higher biological activities (8 fractions for *S. nigrum* and 6 for *C. italica*, as in **Tables 2** and **3**) were chosen for identification using spectroscopic techniques.

3.2. Anticancer activity

Our previous study (Aboul-Enein *et al*., 2012) showed that

Table 1. Antioxidant and anticancer activity % of pure compounds separated from *Solanum nigrum*.

Compound No.	Anticancer activity (against EACC)	Antioxidant activity
1	76	80.5
2	74	65.6
3	72.5	50.5
4	71	65.3
5	74	60
6	68	40.8
7	60	45.6
8	60	30.2
LSD	1.85	1.28

Each value is presented as mean of triplicate treatments. LSD significantly at p#0.01 according to Duncan's multiple range test.

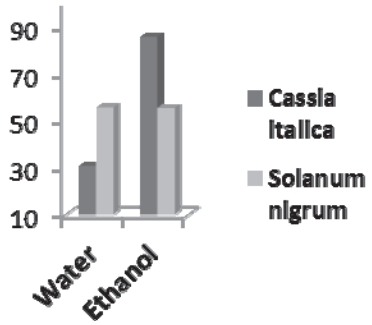

Fig. 1. Antioxidant activity of crude ethanolic extract of Cassia italic and *Solanum nigrum*.

Table 2. Antioxidant and anticancer activity of compounds separated from *Cassia italica*.

Compound No.	Anticancer activity (against EACC)	Antioxidant activity
1	55	48.7
2	57	34.8
3	59	78.4
4	59	30.2
5	50.6	68.4
6	68	56.7
LSD	0.65	2.65

Each value is presented as mean of triplicate treatments. LSD significantly at p#0.01 according to Duncan's multiple range test.

both ethanolic and water extracts of *Cassia italica* and *Solanum nigrum* showed anticancer and antioxidant activities more than 80%. In this work we repeated the anticancer and antioxidant activities of crude extract and the obtained data were nearly the same (**Fig. 2**).

The results obtained from this study represent an important step towards the effective isolation and characterization of the active principles from these plants and to understand the mechanism of cytotoxic activity of these compounds. Data from Table 2 showed that five bands (fractions 1-5) isolated from *S. nigrum* gave anticancer activity against EACC more

Table 3. Anticancer activity of active ingredients (at 50 and 100 ug/ml) against HepG2 cell line and its effect on Caspase enzyme expression.

Compound no.	Concentration (µg/ml)		Caspase enzyme (Pg/ml)
	50	100	
Solanum nigram			
1	50	75.3	3.16
2	9.4	45.1	2.82
3	50	55.6	2.95
4	55.1	78.9	3.61
5	58.7	71.8	3.48
6	63	72.8	2.8
7	56.8	77.6	3.56
8	69	77.2	3.29
Cassia italica			
9	29.8	79.4	2.78
10	44.3	78.65	4.04
11	32.9	59.8	2.87
12	33.5	66.3	3.91
13	43.9	65.8	3.61
14	42.65	69.6	3.5
LSD	1.47	1.96	0.032

Each value is presented as mean of triplicate treatments. LSD significantly at p#0.01 according to Duncan's multiple range test. Data are expressed as mean 6 S.D.

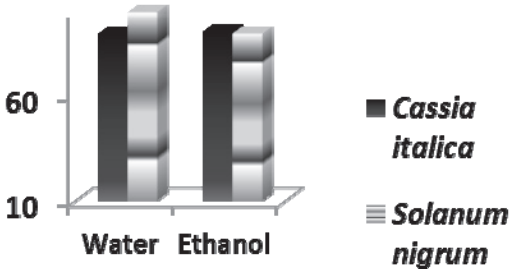

Fig. 2. Anticancer activity of crude ethanolic extract of *Cassia italica* and *Solanum nigrum*.

than 70% while three bands (fractions 6-8) came in the second rank which gave anticancer activity more than 50% (68, 60 and 60%, respectively). Two other bands (fractions 9, 10) gave anticancer activity less than 50% (not given in Table 2). Concerning *Cassia italica*, six bands (fractions 1-6) gave anticancer activity more than 50% while the others gave anticancer activity less than 50% (not given in Table 3).

In a similar study Patel *et al*. (2009) found that both the crude extracts and the isolated components of *S. nigrum* possessed antiproliferative activity on various cancer cell lines. Antiproliferative activities of the crude organic extract and the isolated compounds were studied on tumor cell lines of liver (HepG2), 21, 22 colon (HT29 and HCT-116), 21, 22 breast (MCF-7), 26 and cervical (U1424, 25 and HeLa27). Data in the present study showed that *S. nigrum* extract has significant

cytotoxicity effect on HeLa Cell Line and affect proteases that activate programmed cell death (**Table 3**). These results were in agreement with the results obtained by Riboli and Norat (2003) who suggested that an inverse association exists between consumption of vegetables and fruits and the risk of human cancers at many sites using Epidemiological studies. The aqueous extract of *S. nigrum* contains steroidal glycosides and glycoprotein which exerted an inhibitory effect on cell growth and colony formation (of prostate, breast and colorectal cells) as mentioned by Nawab *et al*. (2011). In addition, other study (Li *et al*., 2008) showed that aqueous extract inhibits growth of cervical carcinoma (U14).

Our data revealed that the action of the active principles separated from the tested plant extracts act as enhancers for apoptotic pathway. This is clear from the results of caspase activity. The data showed that the tested compounds activated the enzyme action (protease) to hydrolyze proteins of cancer cells and encourage the programmed cell death of cancer cells.

3.3. Activity of identified compounds from *S. nigrum*

Spectroscopic analysis of the eight active compounds separated from TLC analysis of *S. nigrum* extract in present study was found in **Figure 3**. These fractions were 2,3 Dihydroxypropyl elaidate, Naphtho [2,1-b]furan-2(1H)-one,decahydro-3a,6,6,9a-tetramethy, 5-Bromosalicylaldehyde, 12-sulfanyldodecanoic acid Usnic acid monoacetate. Trilinolein, Niclofen and 8-Azabicyclo [3.2.1] octane-2-carboxylic acid, 3-hydroxy-8-methyl,(2-endo,3-exo). Concerning the fifth compound (Usnic acid monoacetate), the compound belongs to the phenolic class. The identified compounds in Figure 3 mostly belong to the phenolic compounds and showed high potency to cancer cells. In this concern, similar identification has been done by Chou *et al*. (2011) from *T. candida* and *U. barbata* extracts and determined using HPLC-UV analysis. These extracts contain major phenolic compounds as usnic acid (*U. barbata*) and norstictic acid (*T. candida*). The authors mentioned that Usnic acid has the strongest anticancer activity towards both FemX (human melanoma) and LS174 (human colon carcinoma) cell lines.

Much information is available on the reported inhibitory effects of specific plant phenolic compounds and extracts on mutagenesis and carcinogenesis.

Concerning the sixth compound, Trilinolein has anticancer activity against HepG2 cells. This compound has been identified as one of the active constituents isolated from Panax notoginseng and used widely in traditional Chinese medicine. The effect of trilinolein on the growth of non-small cell lung carcinoma A549 was investigated by Chou *et al*. (2011) who

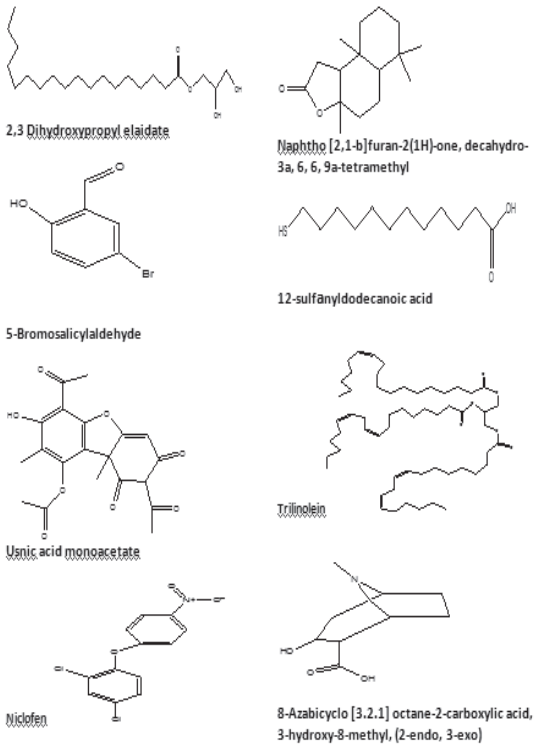

Fig. 3. Suggested chemical structures of identified active compounds isolated from *Solanum nigrum*.

found "that the exposure of A549 cells to trilinolein resulted in the growth inhibition and the induction of apoptosis. The intracellular reactive oxygen species, ROS, seem to play a role in the trilinolein-induced apoptosis. They noticed that ROS produced early in the trilinolein treatment. The most important regulators of trilinolein-induced apoptosis are Bcl-2 family and caspase-3, which are associated with cytochrome C release and dephosphorylation on the Akt signaling pathway."

The anticancer activity of the methanol extract of *Cassia italica* against two types of cancer cell lines, (Hep-G2, Hela cell lines) was also detected (El-Shemy *et al*., 2007). Scientists have suggested that it appears extremely unlikely that any one substance in the extract is responsible for all of the associations seen between plant foods and cancer prevention. This is due to the great variety of dietary phenolics, including flavonoids, and the many types of potential mechanisms reported (Birt *et al*., 2001; El-Shemy *et al*., 2007). These results were in agreement with that given in Table 3 which show that the ethanolic and water extracts of Cassia and *S. nigrum* inhibited the growth of EACC cells in vitro and have antioxidant activity. This appears to involve apoptosis-induced cell loss and a lowering in the proliferation rate of cancer cells.

3.4. Activity of identified compounds from *Cassia italica*

The identification of the six active compounds from *Cassia italica* was performed and the results are given in **Figure 4**.

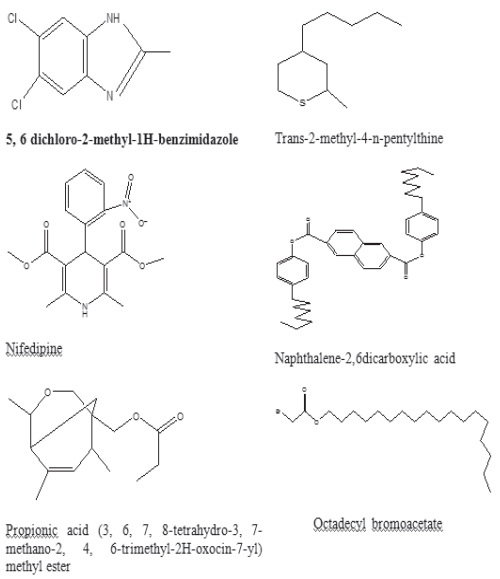

Fig. 4. Suggested chemical structures of identified active compounds isolated from *Cassia italica*.

The six active compounds identified from *Cassia italica* were 5,6-dichloro-2-methyl-1H-benzimidazole, Trans-2-methyl-4-n-pentylthiane, Nifedipine, Propionic acid (3,6,7,8-tetrahydro-3,7-methano-2,4,6-trimethyl-2H-oxocin-7-yl) methyl ester, di-p,octyl phenoxy 1,5 naphthalene dicarboxylase ester, Propionic acid (3,6,7,8-tetrahydro-3,7-methano-2,4,6-trimethyl-2H-oxocin-7-yl) methyl ester and Octadecyl bromoacetate. From the figure, the first compound is 5,6-dichloro-2-methyl-1H-benzimidazole and has high anticancer activity. This is similar to that obtained by Pathak *et al*., (2010) who covered the most active benzimidazole derivatives. These derivatives have shown considerable biological actions such as antimicrobial, anti-inflammatory, antioxidant, anticancer, anticonvulsant, antidepressant, anti-leishmanial and radioprotective. Some new benzimidazole-4,7-diones were synthesized and reported (Gellis *et al*. (2008) which suggest that the compounds perform excellent cytotoxic activity against colon (HT29), breast (T47D) and lung (A549) cancer cell lines and shown lowest IC50 values (3 μM)). Also antioxidant activity by synthesis of some 6-flouro-5-substituted benzimidazole (Ateş-Alagöz *et al*., 2005) showed strong super scavenging effect on superoxide anion at 10^{-3} M concentration. Concerning the third compound Nifedipine, it belongs to 1,4-dihydropyridine (1,4-DHP). The most feasible position for substitution is 4th which exhibit various activities i.e., as the calcium channel antagonists (David and Triggle, 2007).

Swarnalatha *et al*. (2011) also found that "the heterocyclic ring is the common feature for various pharmacological activities such as anti-inflammatory activity (Bahekar and Shinde, 2002), antitubercular activity (Wachter and Davis, 1998), analgesic activity (Gullapalli and Ramarao, 2002), antihypertensive, antianginal (Breitenbucher and Figliozz, 2000) antitumor (Boer and Gekeler, 1995), antithrombotic (Sunkel *et al*., 1990). It binds to L-type channel and also shows action by binding to N-type channel (David and Triggle, 2007)." Nifedipine, a drug used for treatment of hypertension and angina, exerts its effect by calcium channel blockade and nitric oxide production. Arslan *et al*. (1985) measure free cytosolic Ca^{2+}, in the two highly differentiated tumor cell lines, Ehrlich and Yoshida ascites carcinomas, by using a new lipid-soluble heavy metal chelator TPEN. The authors revealed that these ascites tumor cell lines have normal cytosolic free Ca^{2+}. Also, the effects of calcium channel blockers (CCBs) on apoptosis of cancer cells were investigated. CCBs verapamil and diltiazem inhibited apoptosis (Ares *et al*., 1997; Nagashima and Goto, 2000). On the other hand, in some cases, they were also shown to be apoptosis inducers (Balakumaran *et al*., 1996). Concerning the fourth compound, previous reports mentioned that naphthalene derivatives have demonstrated a strong growth inhibitory activity against cell lines (Abdelwahab, 2009). Therefore, these derivatives of naphthalene might be responsible for the anticancer properties of the ethanol extract of *Cassia italica* against EACC cancer cells. The anticancer and antioxidant activities of the plant may be due to the first compound that belongs to benzimidazole derivatives that have shown considerable biological actions such as antimicrobial, anti-inflammatory, anticancer and antioxidant. The enhancing of caspase-3 in cancer cells after incubation with anticancer compounds reveals the apoptosis of these cells.

It is essential to mention that the anticancer and antioxidant activities are associated with the content of phenolics, including flavonoids. Our preliminary studies showed a good relationship between antioxidant potency and the total phenolic content as well as between the antioxidant potency and the reducing power (Abou Elalla and Shalaby, 2009). The total phenolic contents correlated significantly and positively with the activity of antioxidation enzymes. In addition, the anticancer activity was found to be related to alkaloids and phenolics from *C. italica* and *S. nigrum*. The activity may be related only to phenolics from water hyacinths or to glycosides and plant acids from *C. italica* (Trease and Evans, 1989).

All the extracts which gave high anticancer potency have high antioxidant activity while the opposite trend is not observed (Aboul-Enein *et al*., 2012; Abou Elalla and Shalaby, 2009). In this concern, cancer is a multistage process defined by at least three stages: initiation, promotion, and progression (Ames and Gold, 1992; Schulte-Hermann *et al*., 1990). Oxidative stress interacts with all three stages of this process. During the initiation stage, ROS may produce DNA damage by introducing gene mutations and structural alterations into

the DNA. In the promotion stage, ROS can contribute to abnormal gene expression, blockage of cell-to-cell communication, and modification of second messenger systems, thus resulting in an increase in cell proliferation or a decrease in apoptosis of the initiated cell population. Finally, oxidative stress may also participate in the progression stage of the cancer process by adding further DNA alterations to the initiated cell population (Klaunig *et al*., 1998).

As a conclusion, the present study showed that crude extract and the isolated compounds from *Cassia italica* and *Solanum nigrum* leaves have anticancer and antioxidant activities. The results showed that 8 compounds were purified from ethanolic extracts of *Solanum nigrum* whereas 6 extracted from *Cassia italica* which have anticancer and antioxidant activities. There is a good relationship between the antioxidant potency and reducing power. Treatment of the cancer cells with the active compounds led to their growth inhibition and the induction of apoptosis in cancer cells. Intracellular reactive oxygen species seem to play a role in the induced apoptosis.

It is apparent from our study that effective drugs produced from *Solanum nigrum* and *Cassia italica* tend to support the traditional medicinal use of the plants in the treatment of cancer and could lead to development of local pharmaceutical industries, thereby enhancing self-reliance and reducing drug importation.

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