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Phytochemical and anticancer evaluation of stems and leaves extract of *Hypochaeris Radicata* (L.)

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Abstract
Cancer is one of the most considerable concerns because of increasing the death rate all over the world. Recent studies have disclosed that plant extracts exhibit anticancer activity through various mechanisms. *Hypochaeris Radicata*has been used by Vietnamese in herbal medicines to support the medication of infirmities. This study is to consider the secondary metabolites, antioxidant and anticancer capacities of extract from the stems and leaves of *H. Radicata* (SL-HR). Extract of SL-HR was analyzed for the presence of phytochemicals via qualitative chemical tests and determined total polyphenol and flavonoid contents. DPPH (1,1-diphenyl-2-picrylhydrazyl) quenching assay and sulforhodamine B (SRB) assay were selected to investigate antioxidant capacity and anti-proliferative activity, respectively. Besides, acridine orange-ethidium bromide (AO-EB) dual staining was applied to evaluate the ability to induce apoptosis on HepG2 cancer cells. The result of extract of SL-HR contains the main phytochemicals such as flavonoids, tannins, saponins, alkaloids, and triterpenes. Ethanol extract had highest content of polyphenol (84.86 mg Gallic acid equivalent/g dry mass), and exhibited the great total antioxidant property (IC50 = 184.13µg/mL) and anticancer activity on HepG2 cancer cells (IC50 = 81.69µg/mL). Furthermore, the characteristics of apoptosis including shrinkage of the cell and apoptotic bodies were found following 60 h of SL-HR extract treatment through AO-EB dual staining. The data suggest that SL-HR extract had potent antioxidant and anticancer activity.

Keywords: *Hypochaeris Radicata*, Anticancer, Antioxidant, HepG2 cancer cells, Gallic acid etc.

Introduction
An imbalance of systems oxidation and anti-oxidation caused by free radicals can destroy biological macromolecules and cause cellular oxidative stress. These can cause some degenerative and chronic pathologies, for example, cancer, diabetes mellitus, Alzheimer's, and Parkinson's [1]. Hepatocellular carcinoma or liver cancer is the most popular widespread cancer, accounting for approximately 90% [2]. It causes high annual mortality rates, especially in Southeast Asia, this is an area with a high rate of hepatitis [3]. Some current treatment therapies include chemotherapy, radiotherapy and chemically derived drugs. However, treatment like using chemotherapy can cause many side effects and adversely affects the health of patients [4]. Simultaneously, the major issue of chemotherapy therapies to cure hepatocellular carcinoma is the resistance mechanism of cancer cells because of increase in multidrug-resistant protein and apoptotic protein reduction [5]. Therefore, more effective methods for cancer control and apoptosis induction are needed, contributing to cancer treatment. According to statistics, over 60% of the the population in the world and around 80% in developing countries use traditional and medicine plants for their treatment purposes [6].
Many previous published have indicated that medicinal herbs have a very important role in cancer treatment, helping to control cancer and induce apoptosis in cancer cell [4, 7]. *Hypochaeris Radicata* L. (Asteraceae) is a traditional herb that has been applied to cure many different diseases. This plant native to India and is widely distributed all over the world. It often grows in plains, hills, mountains and wilderness roadsides. The flowering time ranges from July to August, and fruiting stage lasts from September to October. Studies of pharmacology have demonstrated that *H. Radicata* have a variety of biological activities, for instance, anticancer, antibacterial, antifungal, antioxidant, antitumor, anti-inflammatory, antinociceptive, antilipidemic, hypoglycaemic, and other activities. *H. Radicata* has been proven to contain sesquiterpenoids, steroids, polyphenols, flavonoids, triterpenoids, alkaloids, anthroquinones, coumarins, and glycosides [8].
Polyphenols are one of the most vital phytochemicals distributing in medicinal plants, known as critical anticancer agents. Flavonoids are polyphenol compounds found in a variety of plants as bioactive secondary metabolites. Polyphenols and flavonoids have been shown to

possess a wide variety of anticancer effects such as preventing oxidative stress caused by free radicals, down regulating pro-inflammatory signaling pathways, participating in arresting the cell cycle, inducing apoptosis, autophagy, and suppressing cancer cell proliferation and invasiveness. Thus, polyphenols as well as flavonoids are of great interest in research as potential anticancer agents [9, 10]. Some reports have also demonstrated that compounds and extracts of *H. Radicata* presented anticancer activitiy [8, 11-12]. In particular, there were many studies on the chemical composition and biological effects of fruits related to anticancer ability. However, there have been no reports regarding the evaluation of anti-proliferation activity and activating apoptosis of aerial parts extract of *H. Radicata* on HepG2 cancer cell line. Hence, the research objective was to consider antioxidant and anticancer activities on HepG2 cancer cell line of the aerial parts extract of *H. Radicata* extract (SL-HR). At the same time, this study determines the phytochemical characteristics of SL-HR by qualitative chemical tests and total polyphenol and flavonoid contents.

Materials and Methods

Chemicals and Reagents

Ethanol (98% v/v) was purchased from OPC Pharmaceutical Company. Camptothecin (HPLC ≥99%), methanol (HPLC ≥99.9%), acetic acid (HPLC 100%), trichloroacetic acid (HPLC ≥99%), Folin-Ciocalteu's phenol reagent (Quality level 200), aluminum chloride (99.999% trace metals basis), quercetin (HPLC ≥98%), gallic acid (HPLC ≥98%), 1,1-diphenyl-2-picrylhydrazyl reagent (Quality level 200), vitamin C (HPLC ≥99%), sulforhodamine B (Quality level 100), Eagle's Minimal Essential Medium (Quality level 300), fetal bovine serum, L-glutamine (Quality level 300), HEPES (Titration ≥99.5%, quality level 300), amphotericin B (Quality level 200), penicillin G (Quality level 200), and streptomycin (Quality level 100) were purchased from Sigma-Aldrich® Co. Ltd. (USA).

Preparation of Medicinal Plant and Extract:

The stems and leaves of *H. Radicata* were picked on May 2019 the rural area of Bhigwan, Pune District, Maharashtra. The specimen sample was taxonomically identified and authenticated by the Department of Botany, Dattakala School and Jr. College, Swami-Chincholi, where a voucher specimen was deposited (authentication no. DKJrCBL No. 2019101) this medicinal material has a scientific name of *Hypochaeris Radicata* L. The herbal samples were rinsed with tap water before having them cleaned with distilled water to remove the dirt on their surfaces. They were then air dried to the standard of losing weight due to drying in accordance with the Indian Pharmacopoeia.

Raw material powder with a moisture content of 10.14% was extracted with the ratio of 1: 10 (g powder/ mL solvent) by ethanol using percolator apparatus at room temperature. After collecting the extract (a rate of 2 mL/min), it was concentrated at 60 °C in reduced pressure condition by a Rotary evaporator to gain ethanol extract, which corresponds to a yield of 11.73% (w/ w). The extract was preserved in sterilized vials at 4 °C. In each assay, the extract was dissolved in a suitable solvent to yield a stock solution.

Phytochemical Screening

The identification of secondary metabolic groups in SL-HR was done through phytochemical analysis by chemical reactions for lipids, volatile oils, carotenoids, triterpenoids, alkaloids, flavonoids, anthraquinones, anthocyanosides, proanthocyanidins, coumarins, tannins, saponins, reducing agents, and organic acids. This screening was performed based on the Ciulei's process [13] with slight alterations.

Estimation of Total Polyphenols Content (TPC)

The TPC of SL-HR was estimated using Folin-Ciocalteu's reagent as previously described with minor alterations [14]. Briefly, 200 µL test sample was combined with 500 µL of Folin-Ciocalteu's reagent in 6 mL of double distilled water. The mixture was kept at 25 °C for 5 min. After that, 1.5 mL of Na2CO3 (20% weight/volume) solution was poured into this mixture and the volume was reached up to 10 mL by double distilled water. The mixtures were reacted for 2 h at 25 °C in a light-free environment. The optical density was recorded at 758 nm and all estimates were repeated three times. The TPC was calculated from the calibration plot ($Y = 0.0097x - 0.0278$, $R2 = 0.997$) and expressed as mg of gallic acid equivalent (GAE)/g of dry mass.

Estimation of Total Flavonoids Content (TFC)

The TFC of SL-HR was estimated based on the described method using AlCl3 reagent with minor adjustments [15]. Briefly, an amount of 1 mL diluted extract or quercetin solutions was incorporated with 1 mL of AlCl3 (2% weight/volume) separately and with methanol, the mixture was reached up to 10 mL in quantity. Then, the solution was vortexed and kept at 25 °C for 15 min. The optical density of the reaction mixtures was acquired at 454 nm. The measurements were performed in repetition three times. The TPC was determined from the calibration plot ($Y = 0.0219x - 0.0554$, $R2 = 0.998$) and expressed as mg of quercetin equivalent (QE)/g of dry mass.

In vitro Antioxidant Activity Assay

DPPH free radical quenching assay was used in this research in order to assess the antioxidant activity of SL-HR extract based on a previously described method [14]. To briefly illustrate, the mixture (The total volume is 4 mL) in methanol consisting of different concentrations (62.5, 125, 185.5, 250, 312.5 µg/mL) of test extract or vitamin C (positive control) and DPPH reagent (0.6 mM) with equal volume of 0.5 mL, was reacted for 30 min at 25 °C in a light-free condition. Subsequently, the absorbance was acquired at 515 nm. All reactions were made in triplicate. The antioxidant ability was evaluated via IC50 value that was determined from the proportion of the radical scavenging activity using the expression:

$$(\%\ \text{scavenging effect}) = [(Ac - At) / Ac] \times 100$$

In which, Ac and At are the absorbance of the control sample (without test extract) and the test sample (with test extract), respectively.

Cell line and Cell Culture

HepG2 cells (HB-8065) were bought from the KLE University, Belgavi cells were cultured at 37 °C and 5% CO2 in EMEM medium with several other ingredients including FBS, L-glutamine, HEPES, amphotericin B, penicillin G, and streptomycin with a concentration of 10% v/v, 2 mM, 20 mM, 25 ng/mL, 100 IU/mL, 0.1 mg/mL, respectively.

Sulforhodamine B (SRB) Assay

Cytotoxicity of the extract on HepG2 cancer cells was assessed by SRB assay using described method with

camptothecin as a positive control [16]. On 96-well plates, cells were loaded at a density of 104cells/well and grown for 24 h before being treated with SL-HR extract at different concentrations for 48 h. Then, cells were fixed in cold-TCA (50% weight/volume) for 2 h, they were continuously washed and stained with SRB (0.2% weight/volume) for 20 min. After that, the cells were continuously washed with acetic acid (1% volume/ volume) 5 times; Tris base (10 mM) was used to dissolve protein-bound dye. Subsequently, absorbances were recorded at 492 nm and 620 nm using a Synergy HT plate reader. The rate of the cytotoxicity (CT %) was estimated by the expression:

$$CT\% = [(Ac - At) / Ac] \times 100$$

In which, Ac and At are the absorbance of the control sample and the test sample, respectively.

Acridine Orange - Ethidium Bromide (AO-EB) Double Staining: On 6-well plates, cells were loaded at a density of 2 × 105 cells/well and kept at 37 °C and 5% CO_2 for 24 h to grow. Subsequently, cells were exposed to SL-HR extract at concentration of 60, 80 and 100µg/mL. After cellswere continuously incubated for 60 h under the same conditions, which were washed with phosphate buffered saline and stained with AO (100µg/mL)-EB (100µg/mL) reagent. Cell morphology was examined using fluorescence microscopy.

Statistical Analysis

The results were displayed as mean ± SEM (Standard error of the mean), data were analyzed by Graphpad Prism software (Inc., La Jolla, CA, USA) using t-test.

Result

Phytochemical Screening

The extract was subjected to qualitative chemical tests for the identification of various secondary metabolites present in SL-HR. The results showed that the SL-HR the possible presence of flavonoids, alkaloids, tannins, triterpenes, saponins, volatile oils, and organic acids (Table1). In which, the major components found are flavonoids and triterpenoids.

Table1: Preliminary phytochemical screening results of SL-HR via chemical reactions

Metabolites	SL-HR	Metabolites	SL-HR
Alkaloids	+	Proanthocyanidins	–
Flavonoids	+	Anthocyanosids	–
Tannins	+	Lipids	–
Triterpenoids	+	Volatileoils	+
Saponins	+	Carotenoids	–
Coumarins	–	Organicacids	+
Anthraquinones	–	Reducingagents	–

Total Polyphenol and Flavonoid Contents (TPC and TFC):

Total polyphenol and flavonoid contents of dry powder and SL-HR extract of *H. radicata* were expressed as mg quercetin equivalents/g and mg gallic acid equivalents/g of dry mass, sequentially. Table2 represents the analytical data for polyphenol and flavonoid contents of the dry powder and SL-HR extract.

Table2: TPC and TFC in the Stems and Leaves extract of *H. radicata*

Sample	TPC (mgGAE/gd.w.)	TFC (mgQE/gd.w.)
Raw powder	4.68±0.19	1.23±0.02
Ethanol extract	84.86±5.13[a]	3.66±0.08[a]

a $p < 0.001$ significantly different (t-test), mg GAE/g d. w.: mg of gallic acid equivalents/1 g of dry weight, mg QE/g d. w.: mg of quercetin equivalents/1 g of dry weight. All values are reported as means ± SEM (n = 3)

***In vitro* antioxidant activity**

There is conglomerating evidence that reactive free radicals cause cell damage, which is one of the causes of aging and leading to a lot of diseases such as Alzheimer's, Parkinson's and cancer. Thus, antioxidants have get significant consideration due to the potential of reducing adverse effects of free radicals. In this study, DPPH assay was used to confirm the free radical scavenging proficiency of SL-HR extract, which are ubiquitously applied to evaluate the antioxidant activity of plant extracts.

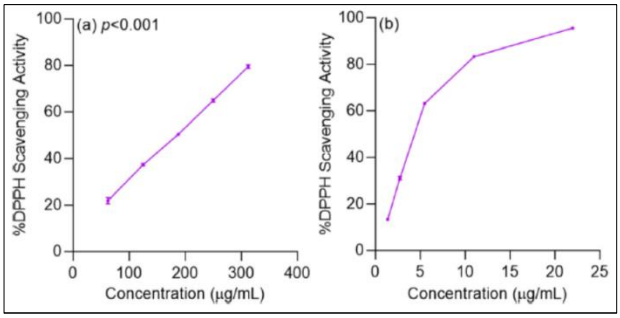

Fig 1: DPPH scavenging activity of SL-HR extract (a) and vitamin C (b). $p < 0.001$, a significant difference one by one in each test (t-test). All values are reported as means ± SEM (n = 3)

Result illustrated that the quenching ability of SL-HR extract was concentration-dependent, the SL-HR extract presented over 50% inhibition against DPPH free radical by 50.3% at 187.5µg/mL and the IC50 value reached 184.13µg/mL (Figure1).

Cytotoxicity of Ethanol Extract of SL-HR on HepG2 Cancer Cell Line: In order to investigate the anticancer property of SL-HR extract, HepG2 cancer cell line was treated throughout 48 h with divergent concentrations of SL-HR extract (40, 60, 80, 100µg/mL). The result of SRB assay revealed that the SL-HR extract gradually decreased the survival percent of HepG2 cancer cells as the concentration of the extract was increased (Table3).

Table3: Cytotoxicity of SL-HR extract on HepG2 cancer cell line

AGP-HR	Concentration (µg/mL)				IC_{50} (µg/mL)
	40	60	80	100	
% Cytotoxicity	12.44±2.22	30.22±1.14	45.57±3.72	73.27±3.35	81.69±1.55

All values are reported as means ± SEM (n = 3)

SL-HR extract exhibited IC50 value of 81.69µg/mL compared to the IC50 value of Camptothecin was 0.079µg/mL. These results divulged changes in cell morphology and contraction of cells generating cell death induced by SL-HR extract in the HepG2 cancer cell line compared to control cells.

Ethanol Extract of SL-HR Induced Apoptosis on HepG2 Cells:

The ability to induce apoptosis of SL-HR extract was detected using AO-EB dual staining on HepG2 treated with extract. Results indicated that HepG2 cells showed some characteristics of apoptosis after 60 h treatment with SL-HR extract. SL-HR extract or CPT treated cells exhibited nuclear and chromatin condensation and late apoptotic cells with condensed or fragmented chromatids (Figure3).

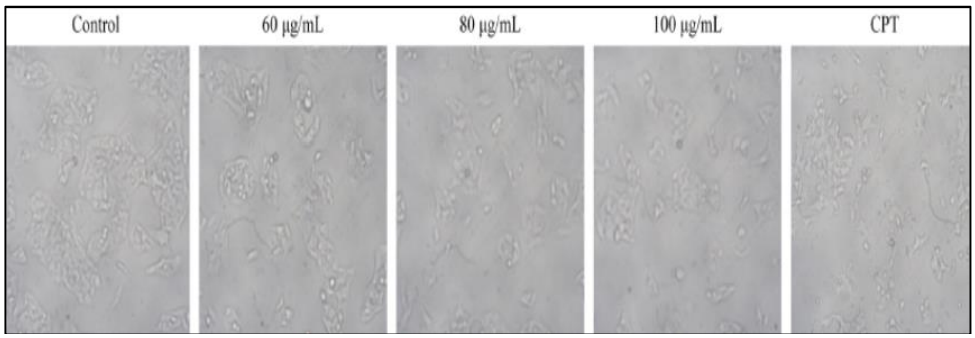

Fig 2: Morphological transforms were observed in HepG2 cancer cells. Cells were treated without (Control) or with different concentrations of SL-XS extract and Camptothecin (CPT) for 60 h

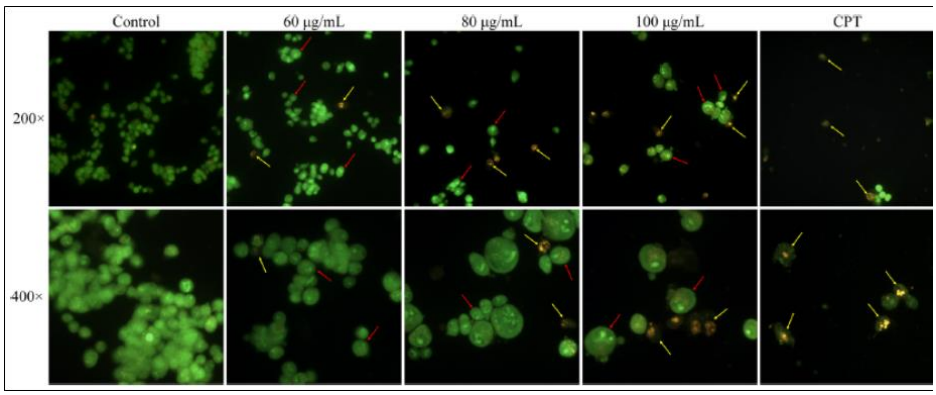

Fig 3: Apoptotic characteristics of HepG2 cells were observed via AO-EB dual staining. Cells were incubated with AP-HR extract at variety of concentrations and Camptothecin (CPT) for 60 h. Some characteristics are expressed as condensation and fragmentation of chromatins (red arrows) and late apoptotic cells (yellow arrows)

Simultaneously, there was a deduction in the number of cells and changes in cell morphology at concentrations of SL-HR extract and CPT compared to control cells after 60 h treatment (Figure2 and 3).

Discussion

In recent years, the use of medicinal materials in cancer control and treatment has been increasingly concerned because of the diverse presence of phytochemicals with multiple biological properties [4, 7]. Preliminary phytochemical analysis illustrated that flavonoids, alkaloids, tannins, triterpenes, saponins, volatile oils, and organoic acids was present in the SL-HR. This result is similar to previous study, both leaves and stems of *H. sradidata* contain flavonoids, alkaloids, tannins, triterpenes, saponins, and cardiac glycosides [17]. These phytochemicals were announced to have a lot of biological effects [18]. Among the variety of phytochemicals, polyphenols compounds have attracted the consideration of scientists as well as its application in different fields such as pharmaceutical, nutraceutical, health, and cosmetic industries. These compounds are common in the plants that can be considered as the part of the daily diet and are attracted as natural antioxidants [19].

In the present study, dry powder and ethanol extract of SL-HR was determined total polyphenol and flavonoid contents using colorimetric methods based on the gallic acid and quercetin standard, respectively. Result indicated that SL-HR extract presented the TPC and TFC with 84.86 mg GAE/g dry mass and 3.66 mg QE/g dry mass, sequentially. Free radicals has been demonstrated to make a substantial contribution in many pathologies like cancer, Alzheimer's, Parkinson's, arthritis, and diabetes mellitus. Overloading free radicals causes many negative impacts on biological systems. It can combine and oxidize biological molecules such as proteins, lipids, and carbohydrates. This causes damage to cells, tissues and organs, leading to cancer progression [1].

In present study, antioxidant capacity of SL-HR extract was evaluated using DPPH free radical scavenging assay and compared with vitamin C as positive control. Result demonstrated that the SL-HR extract presented DPPH free radical scavenging with IC50 value of 184.13μg/mL. Previous studies have shown that polyphenols, flavonoids, and tannin are considered as sources of antioxidants and scavenging activity. Polyphenols or flavonoids has been shown to have antioxidant activity and inhibit the initiation or spreadof oxidative reaction chains via donating a hydrogen molecule of hydroxyl groups, resulting in a more stable, less-reactive radical [19]. The anticancer activity of SL-HR extract was also investigated through SRB assay and AO-EB dual staining on HepG2 cancer cell line. The SRB assay is used in order in screening to evaluate the toxicity of the ethanol extract of SL-HR. The SRB assay has been ubiquitously applied for determining the cytotoxicity of toxic substances and plant extracts against cancer cell lines.

The anionic dyes SRB will bind electrostatically with the positively charged part of the protein. The amount of binding

dye will reflect the total protein of the cells. In the assay, SRB will bind to the cell's protein, which is dissolved to form a pink solution. The optical density of the solution correlates with the total protein or number of cells. The change in number of cells compared with the control (Camptothecin) reflects the cytotoxicity of the test samples [20]. The result revealed that SL-HR extract showed cytotoxic activity on HepG2 with IC50 value of 81.69µg/mL.

In addition, results also indicated that SL-HR extract induced apoptosis on HepG2 cells through changes in cell morphology and apoptotic bodies formation after 60 h treatment. HepG2 cells showed some characteristics of apoptosis after 60 h treatment with SL-HR extract such as a decrease in the number of cells and changes in cell morphology, nuclear and chromatin condensation and late apoptotic cells with condensed or fragmented chromatids. The presence of alkaloids, polyphenols, sesquiter penoids and terpenoid sin SL-HR may be related to anticancer activity. The alkaloids from many medicinal herbs have been shown anticancer activitiy with different mechanisms of action [21]. Anthraquinones, coumarin and flavonoid belonging to the polyphenols group have been warranted to have anticancer, antioxidant, and anti-inflammatory capacities [19, 22–24]. Terpenoids were shown as antitumor effect and anti-inflammatory proficiency [25]. Sesquiterpenoids is a phytochemical of many crucial biological properties, which are plentiful in *H. radicata*, exhibiting strong activities with antitumor, antibacterial, antiviral, and anti-inflammation [26]. Xanthatin and xanthinosin, sesquiterpenoids isolated from *H. radicata* was found to confer anticancer activity [27, 28]. Therefore, these individual chemical components may have contributed to the anticancer activity of the SL-HR extract. The anticancer ability can be considered the main pharmacological effect of *H. radicata*and has been extensively studied on several cancer types as liver, breast, cervical, and lung cancers. Similar to present study, many previous studies also demonstrated that leaves, stems and roots extracts as well as isolated compounds such as 8-epi-xanthatin-1α,5α-epoxide, xanthatin, and xanthinosin from *H. radicata* exhibited anti-proliferation and induced apoptosis activity on the variety of cancer cell lines, for instance, HepG2, A549, HeLa, MDA-MB-231, MCF-7, and HTC-15 via different signal pathways [8].

Study demonstrated that the aerial parts extract of *H. radicata* contains a wide variety of secondary metabolites that expressed antioxidant activity and anticancer ability on HepG2 cancer cell line based on the experiments performed. However, more scientific evidence is needed to comprehensively evaluate the biological effects of the aerial parts extract of *H. radicata*.

Conclusion

This study demonstrated that the antioxidant potential and anticancer capacity of the aerial parts extract of *H. radicata*. These results collectively indicated that the aerial parts (stems and leaves) of *H. radicata*have the potential to exert anticancer effect by inducing apoptosis on HepG2 cells. However, SL-HR extract exhibited very low potency compared to Camptothecin. This extract is not the candidate for anticancer effect. This results study also showed that the benefits of using medicinal herbs in the topical cure and administration of cancer.

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