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In Vitro Evaluation of Anti Cancer and Antioxidant Activities of Siddha Polyherbal Formulation – Maramanjal Chooranam

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ABSTRACT: Evaluation of anticancer and antioxidant activities has been screened by Maramanjal Kudineer Chooranam. Decoction, extracts will be investigated for their total flavonoids, phenol contents and their antioxidant activity of ABTS 2,2′-azino-bis (3-ethyl benzothiazoline-6-sulfonic acid), Lipid peroxidation, metal chelating, Superoxide scavenging assay and invitro anticancer activity. The total phenolic and flavonoid content of extracts will be determined by using FC (Folin–Ciocalteu) and $AlCl_3$ colorimetric assay method. Anticancer activity will be screened for MCF-7 breast cancer cell line. Decoction, extracts has been determined significant of antioxidant activity and anticancer activity compared with standard (BHT) Butylated Hydroxy Toluene. These Decoction, extracts of "Maramanjal Kudineer chooranam" has been considered as a natural source for using antioxidant, and anticancer agents compared to commercially available synthetic drugs.

Keywords: Maramanjal Kudineer Chooranam, antioxidant, anticancer.

INTRODUCTION

Oxidants are groups of atoms or an uncharged atom with minimum of one unpaired electron (free radical) in the outer most orbit; therefore, they are highly reactive. In living membranes, they cause cellular disintegration, which ultimately leads to death. They are usually formed by various chemicals that are present in the environment such as polluted air, water, etc. and are prevalent in large numbers in the blood. To counteract the negative effects of these radicals, the living system produces numerous antioxidants. If the living system is unable to do, supplements must be added in the diet. Their mode of action involves polyunsaturated lipids (lipid peroxidation) of cell membranes, along with the standard RNA and DNA of the cell. An active antioxidant compound has several radical gathering antioxidant sites of varied nature. These sites seek and terminate free radicals at many cellular sites (Warner *et al.,* 2004).

Humans when subjected to any kind of stress are reported to produce more reactive oxygen species (ROS). Some of the ROS popularly found in the cell include superoxide anion radicals, hydroxyl radicals and hydrogen peroxide. In such a scenario, the number of ROS is more than enzymatic antioxidants (e.g., catalase and glutathione peroxidase (GPx), as well as superoxide dismutase (SOD) and non-enzymatic antioxidants (e.g., ascorbic acid, α-tocopherol and glutathione). Because of this disproportion in number, there is no sufficient amount of antioxidants to quench the free radicals and the cells are damaged (Peuchant *et al.,* 2004) which can result in serious health issues (Steer *et al.,* 2002). A paucity of antioxidants could expedite the onset of neurodegenerative diseases, Alzheimer's disease (Di Matteo and Esposito, 2003), degenerative diseases and cancers (Gerber *et al.,* 2002), cardiovascular diseases and inflammatory ailments (Sreejayan and Rao 1996). As plants are easily accessible source of antioxidants, they serve as protective medicine (Knekt *et al.,* 1996). Accordingly, nearly 80% of the population depend on traditional medication for their primary healthcare needs (World Health Organisation, 2004). Most of the demand is met by the usage of herbs and their bioactive molecules (Winston, 1999).

Numerous antioxidant molecules occur naturally in plants as active oxygen scavengers (Brown and Rice-Evan 1998). Fruits such as citrus, berries, cherries and prunes are teeming with antioxidants. Scientists claim that individuals on a plant-based diet, with increased intake of any edible part of plants, have lesser risk of developing oxidative stress-related diseases. These findings have been further substantiated by Aqil *et al.* (2006), who highlighted that the antioxidant properties of herbal plants was because of the presence of flavonoids and related compound such as flavones, isoflavones, and other groups of polyphenol molecules

including lignans, anthocyanin, catechins, isocatechins and coumarin.

Among all types of cancers, melanoma is considered most life–threatening. About one in five affected succumb to the disease. Many are affected by some form of malignant tumor during their lifetime (Chhajed *et al.,* 2012). Normal human diploid cells proliferate only for a few generations, thereafter entering into a specific state of replicative senescence. However, cancer cells proliferate forever (Jemal *et al.,* 2011). Surgery is considered to be the most promising technique for removing evident tumors. However, the process may also leave smaller nests of malignant cells in the patients. These remainder cells continue to proliferate harming the patients. Other forms of treatment such as radiation is inaccurate because it is not localized and kills both normal cells and cancer cells, with many lethal side effects (Vaiyapuri *et al.,* 2016).

Breast carcinoma is very common among women. The World Health Organization (2015) had reported 1,150,000 cases of breast cancer worldwide on an annual basis. Epidemiological reviews have shown that women with a family history of malignancy are more at risk, which leads to a reproducible forecaster of breast cancer risk (Palmer *et al.,* 2009). Some studies have also considered obesity as a risk factor. Eventhough several research had been done on the disease, molecular events that lead to its origin have not been understood yet. Furthermore, studies have shown 4–10% of breast cancer to arise as a result of hereditary factors (Honrado *et al.,* 2005).

MATERIALS AND METHODS

Plant materials

Ingredients:

Stem of *Coscinium fenestratum* - 2 part

Root bark of *Toddalia asiatica* - 1 part

Root of *Picrorhiza kurroa* - 1 part

Dosage : 1gm (BD)

Adjuvant : Hot water

Indication : Putru Noi (Cancer)

Collection of Medicinal Plants

The above mentioned sample were obtained from the Siddha garden at the Department of Medicinal Botany, Government Siddha Medical College, Arumbakkam, Tamil Nadu, India.

Phytochemical Screening. The Maramanjal Kudineer Chooranam decoction were extracted in an aqueous solution, for identification of phytochemicals. This was done according to the procedures illustrated by Allen (1974); Harbone (1976). The analysis helped to determine the presence of secondary metabolites such as alkaloids, flavonoids anthocyanins, glycosides, tannins, saponins, terpenoids and polyphenols.

Thin Layer Chromatography (TLC) Study in Maramanjal Kudineer Chooranam decoction. Thin layer chromatography of the Maramanjal Kudineer Chooranam decoction was executed using standard procedures (Markham, 1975). The aqueous methanol extract was placed carefully in precoated aluminum silica gel 60 F, Merck F_{254} using a microcapillary tube. The spots were allowed to dry for few minutes and TLC plate was placed in the solvent mixture (Toluene, Acetone and Formic acid in the ratio of 6:6:1). After running the experiment, the TLC plates were dried and observed under UV at 240 nm and 360 nm in a UV TLC viewer. The Rf value of the spots was calculated by using the standard formula,

Distance travelled by solute

Distance travelled by solvent

***In Vitro* Assay for Cytotoxicity Activity (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT) Assay).** MTT assay helped to determine the anticancer activity of the samples on MCF7 (Mosmann, 1983). Cells were transferred to a 96-well plate at about 1×10^5 cells per well in 0.2 mL of medium. For the next 72 hours, cell plates were incubated in a 5 % CO_2 incubator. The isolated compound was added in varied concentrations in 0.1% DMSO (Dimethyl sulphoxide) and placed in a 5% CO_2 incubator for 24 hours. The sample solution was removed and 0.5% 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT) in phosphate buffered saline was added at 20µL/well (5mg/mL). 1mL of Dimethyl sulfoxide (DMSO) was added after 4hrs of incubation. The viable cells were obtained, and the absorbance was read at 540 nm. The Inhibitory Concentration (IC50) values of the compound was determined through a graph where increase in concentration reduced the cell viability percentage by 50%. The percentage (%) of cell viability was calculated by the following formula:

Percentage of cell viability = A_{540} of treated cells /A_{540} of control cells × 100%

ABTS (2,2'-Azino-Bis-3-Ethyl Benzthiazoline-6-Sulphonic Acid) Radical Scavenging Assay. ABTS radical scavenging activity of Maramanjal Kudineer Chooranam. Decoction were estimated by the method of Re *et al.* (1999). ABTS radical cation ($ABTS^+$) was freshly prepared. This was done by dissolving 5mL of 14 mM ABTS solution in 5mL of 4.9 mM potassium persulfate ($K_2S_2O_8$) solution. The mixture was placed in the dark for 12-16 hours at room temperature. Thereafter, it was ready for use. This reaction mixture was diluted with distilled water to obtain an absorbance of 0.70 ±0.05 at 734 nm. This diluted reaction mixture was used for the antioxidant activity. To the test tubes, both test and standard, 950 µL of ABTS solution was added. The different concentrations of methanol and ethyl acetate extracts (25, 50, 75 and 100 µL/mL) were added to the test group, whereas 50 µL of ascorbic acid was added to the standard. The reaction mixture of both test and standard were vortexed thoroughly for few minutes. The mixtures were then incubated at room temperature for 6 minutes. The absorbance was read at 734 nm by using ELICO (SL150) UV–Vis Spectrophotometer. Vitamin-C was used as reference antioxidant compound. The percentage of ABTS scavenging activity was calculated as;

% ABTS radical scavenging activity = $(A_0\text{-}A1)/A_0 \times 100$

Where A_0 is the absorbance of the control and $A_1$is the absorbance in the presence of the sample extract or standard.

Inhibition of Lipid Peroxidation Activity. Lipid peroxidation was induced by Fe^{2+} ascorbate system in egg yolk according to the method of Bishayee and Balasubramaniyam (1971). The activity was estimated as Thiobarbituric acid reacting substances (TBARS) according to the method of Ohkawa *et al.* (1979). The reaction mixture contained egg yolk 0.1 mL (25% w/v) in Tris-HCl buffer (20mM, pH 7.0); Potassium chloride (KCl) (30mM); Ammonium iron (II) sulphate ($FeSO_4$ $(NH_4)_2SO_4.6H_2O$) (0.06mM); and various concentrations of methanol and ethyl acetate extracts (25, 50, 75 and 100 µL/mL) from the Maramanjal Kudineer Chooranam decoction in a final volume of 0.5 mL. The reaction mixture was incubated at 37°C for 1 hour. After the incubating, 0.4 mL was removed and treated with 0.2 mL Sodium Dodecyl Sulphate (SDS) (1.1%), 1.5 mL thiobarbituric acid (TBA) (0.8%) and 1.5 mL acetic acid (20%, pH 3.5). The total volume was made up to 4.0 mL with distilled water. The mixture was kept in a water bath at 95 to 100°C for 1 hour. After cooling, 1.0 mL of distilled water and 5.0 mL of n-butanol and pyridine mixture (15:1 v/v) were added to the reaction mixture. The mixture was shaken vigorously and centrifuged at 4000 rpm for 10 minutes. The butanol–pyridine layer was removed and its absorbance measured at 532 nm (ELICO (SL150) UV–Vis Spectrophotometer) to quantify Thiobarbituric acid reacting substances (TBARS). Inhibition of lipid peroxidation was determined by comparing the optical density (OD) of treated sample with that of the control. Vitamin-C was used as the standard. Inhibition of lipid peroxidation (%) by the extract was calculated according to the following formula: 1- (E/C) × 100, where C is the absorbance value of the fully oxidized control and E is the absorbance of the test sample.

Metal Chelating Activity. Metal chelating capacity of Maramanjal Kudineer Chooranam decoction were evaluated according to the method described by Iihami Gulcin *et al* (2003). Different concentrations of extracts (25, 50, 75 and 100 µL/mL) were added to 0.05 mL of 2 mM Ferric chloride ($FeCl_3$) solution and 0.2 mL of 5 mM Ferrozine. The mixture was shaken vigorously and allowed to stand for 10 minutes. Thereafter, the absorbance of the solution was measured at 562 nm against a blank. All readings were obtained in triplicate. Vitamin-C was used as the standard. The % inhibition of ferrozoine-Fe^{2+} complex was calculated by following equation:

% Inhibition of ferrozoine- Fe^{2+} complex = $[(A_0-A_1)/A_0] \times 100$

where A_0 was the absorbance of control and A_1 was the absorbance of different solvent extract.

Nitric Oxide Radical Scavenging Activity. Nitric oxide radical scavenging capacity of Maramanjal Kudineer Chooranam decoction was measured according to the method described by Olabinri *et al.* (2010). 0.1 mL of sodium nitroprusside (10mM) was added to phosphate buffer (0.2 M, pH 7.8) and mixed with different concentrations of Maramanjal Kudineer Chooranam decoction. The mixture was incubated at room temperature for 150 minutes. After incubation, 0.2 mL of Griess reagent (1% Sulfanilamide, 2% Phosphoric acid and 0.1% N- (1- Naphthyl) ethylene diamine dihydrochloride) was added. The absorbance of the reaction mixture was read at 546 nm against a blank. All readings were taken in triplicate, and Vitamin-C was used as the standard. The percentage (%) of inhibition was calculated by the following equation:

% Nitric oxide radical scavenging capacity= $[(A_0-A_1)/A_0] \times 100$ where

A_0 was the absorbance of control and

A_1 was the absorbance of the different solvent extracts.

Superoxide Radical Scavenging Assay. Superoxide radical scavenging was determined by measuring the capacity of the Maramanjal Kudineer Chooranam decoction to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) (Fedovich, 1976) in the presence of the riboflavin-light-NBT system. Each 3 mL reaction mixture contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 2 µM riboflavin, 100 µM EDTA, NBT (75 µM) and different concentrations (25, 50, 75 and 100 µL/mL) of Maramanjal Kudineer Chooranam decoction. This reaction mixture was placed in front of fluorescent light for 6 min. Absorbance was measured at 560 nm using an ELICO (SL150) UV–Vis Spectrophotometer. Similarly, identical tubes with reaction mixture placed in dark served as blanks. The percentage inhibition of superoxide generation was measured by comparing the absorbance of the control with test sample solution by the following formula:

Percentage (%) of Super oxide radical scavenging capacity= $[(A_0- A_1)/A_0] \times 100$

where, A0 was the absorbance of control and

A1 was the absorbance of organic solvent extract or standard.

Statistical analysis. The one way analysis of variance (ANNOVA) was used to find out the statistically significant differences between control and treated samples for all the biological activities followed by Tukey test for multiple comparisons and Dunnett's tests (2-sided) has been done to determine which differences were significant.

RESULT

Phytochemical Screening. The phytochemical screening of Maramanjal Kudineer Chooranam decoction studied presently showed the presence of alkaloids, flavonoids, phenol, Terpenoids, tannin and saponin, and absence glycosides (Table 1).

Table 1: Phytochemical screenings of Maramanjal Kudineer Chooranam decoction.

Sr. No.	Phytochemical Constituents	Observation	Maramanjal Kudineer Chooranam decoction
1.	**Alkaloids** -Dragendorff's test -Mayers test	Orange / red precipitate Cream precipitate	+ +
2.	**Flavonoids** -Alkalai Reagent -Lead aceate test	Intense yellow colour Precipitate formed	+ +
3.	**Glycosides** -Keller-Killiani test	Pink colour (Ammonia layers)	+
4.	**Tannin** -$FeCl_3$ test	Blue-black colour	-
5.	**Saponins** -Frothing test	Foam	+
6.	**Terpenoids** **-**Salkowski test	Reddish brown colour ring formed in interface	+
7.	**Polyphenols** **-**Ferrozine test	Raddish blue	+
8.	**Anthocyanin** **-Ammonia test**	Pink color in ammonia layer	+

+ Positive result; - Negative result

The Partial characterization of Maramanjal Kudineer Chooranam Decoction by TLC. The Maramanjal Kudineer Chooranam decoction loaded on Pre-coated TLC plates (60 F_2 54 Merck) and developed with a solvent system of petroleum ether, chloroform and methanol in the ratio of 1:0.5:0.1 were efficient to extract the anti-oxidant and anti-cancer compound it is used for further studies. The developed plate was viewed under UV 240nm and 360nm (Table 2 and Fig. 1).

Table 2: Partial characterization of Maramanjal Kudineer Chooranam decoction by TLC.

Sr. No.	Maramanjal Kudineer Chooranam decoction	
	UV 240 nm Rf value	UV 360 nm Rf value
1.	0.92	0.92
2.	0.87	0.89
3.	0.82	0.87
4.	0.78	0.82
5.	0.71	0.78
6.	0.63	0.71
7.	-	0.63

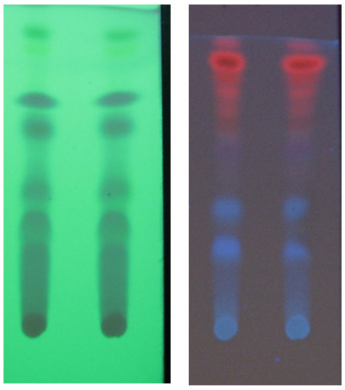
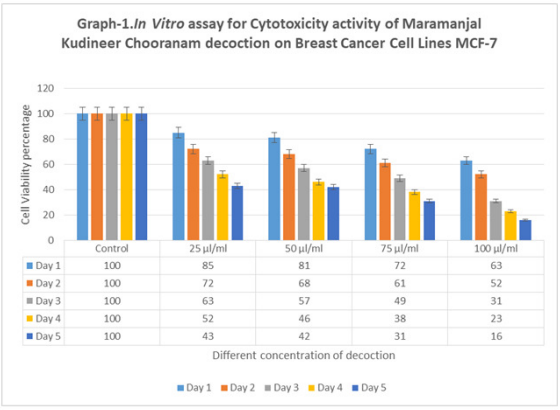


Fig. 1. Partial characterization of Maramanjal Kudineer Chooranam decoction by TLC.

***In Vitro* assay for Cytotoxicity activity of Maramanjal Kudineer Chooranam decoction on Breast Cancer Cell Lines MCF-7**. The cytotoxic effect of Maramanjal Kudineer Chooranam decoction was treated against breast cancer cell lines MCF-7. This was determined by a rapid colorimetric method using MTT (Methyl-thiazolyl-tetrazolium bromide) assay. The OD valoue were recorded and the concentration required for a 50% inhibition of viability (IC_{50} value 35.62) was determined graphically (Graph 1). The effect of the samples on the proliferation of MCF-7 cells was expressed as the percentage cell viability. The results indicated that 16% cell viability was observed against human breast cancer cell line MCF-7 in 100µL concentration of Maramanjal Kudineer Chooranam decoction.



ABTS free radical scavenging activity of isolated compounds. The ABTS radical cation decolourisation test is another widely established approach adopted to evaluate antioxidant activity. Colour reduction shows the decrease of ABTS radical. The Maramanjal Kudineer Chooranam decoction demonstrated

comparatively stronger antioxidant activity as compared to Vitamin-C. The EC_{50} values for ABTS scavenging of isolated compound and standard Vitamin-C were found to be 59.32 µg/mL, 64.32 µg/mL, which indicate the efficient ABTS scavenging activity respectively (Table 3). The Maramanjal Kudineer Chooranam decoction had demonstrated the highest radical scavenging activity when it reacted with the ABTS radicals. This is in keeping with the idea that the flavonoid can generate extra hydrogen radicals due to the greater number of free hydroxyl groups, capable of quenching radicals. Significant differences among the EC_{50} values of isolated compound and Vitamin-C had also been noted ($P < 0.05$).

Inhibition of Lipid Peroxidation Activity. Maramanjal Kudineer Chooranam decoction also inhibited the lipid peroxidation induced by ferrous sulfate in egg yolk homogenates. Maximum inhibition was recorded in Maramanjal Kudineer Chooranam decoction 72.34% with EC_{50} value 65.32µl/ml and lowest inhibition percentage ascorbic acid 68.32% with EC_{50} 68.32 µl/ml (Table 4). As it is identified that lipid peroxidation is the net result of any free radical attack on membrane and other lipid components present in the system, the lipid peroxidation may be enzymatic (Fe/NADPH) or non-enzymatic (Fe/ascorbic acid). In the present study, egg yolk was used as substrate for free radical mediated lipid peroxidation, which is a non-enzymatic method. Normally, the mechanism of phenolic compounds for antioxidant activity includes neutralizing lipid free radicals and preventing decomposition of hydroperoxides into free radicals.

Superoxide Scavenging Assay Activity. Maramanjal Kudineer Chooranam decoction exhibited powerful scavenging activity for superoxide radicals in a concentration dependent process than positive control. Maramanjal Kudineer Chooranam decoction showed highest radical activity in the percentage of 78.32% with EC_{50} value 57.32 µl/ml when compared to positive control 73.64% with EC_{50} Value 62.34 µl/ml (Table 5). One of the standard method to produce Superoxide radicals is through photochemical reduction of nitro blue tetrazolium (NBT) in the presence of a riboflavin-light-NBT system. These superoxide radicals are extremely toxic and may be produced either through xanthine activity or through mitochondrial reaction. Superoxide radicals are reasonably a weak oxidant may decompose to form stronger reactive oxidative species, such as singlet oxygen and hydroxyl radicals.

Metal Chelating Activity. The metal chelating property of Maramanjal Kudineer Chooranam decoction was displayed as per Table 6. The Polyphenol rich fraction form leaves of *Justicia simplex* was evaluated for their ability to compete with ferrozine for ferrous iron in the solution. In this evaluation, the Maramanjal Kudineer Chooranam decoction hindered the formation of ferrous and ferrozine complex, signifying that they have chelating activity and are capable of capturing ferrous iron before ferrozine. Maramanjal Kudineer Chooranam decoction reduced the greenish blue color complex immediately and showed the highest chelating activity 73.64% With EC_{50} Value 64.31 µl/ml than positive control Vitamin-C 67.32% with EC_{50} value 67.23µl/ml.

Table 3: Free radical-scavenging ability using ABTS assay of Maramanjal Kudineer Chooranam decoction.

Different concentration of extract	Percentage of ABTS radical activity	
	Maramanjal Kudineer Chooranam decoction	Standard Vitamin-C
25 µl/ml	23.64 ± 1.87	18.32± 1.79
50 µl/ml	37.32 ± 2.37	32.64 ± 2.78
75 µl/ml	53.21 ± 1.79	48.37 ± 2.34
100 µl/ml	76.38 ± 2.89	71.59 ± 1.89
EC_{50} value	59.32	64.32

[a]Results are expressed as percentage inhibit of ABTS ability with respect to control. Each value represents the mean+SD of three experiments

Table 4: Inhibition of lipid peroxidation activity of Maramanjal Kudineer Chooranam decoction.

Different concentration of extract	Lipid peroxidation inhibition percentage	
	Maramanjal Kudineer Chooranam decoction	Standard Vitamin-C
25 µl/ml	20.35±1.98	18.32 ± 2.54
50 µl/ml	38.21 2.39	35.64± 0.82
75 µl/ml	51.37±1.87	47.32 ± 1.96
100 µl/ml	72.34 ± 2.34	68.32 ± 2.34
EC_{50} value	65.32	68.32

[a] Results are expressed as percentage inhibit of lipid peroxidation with respect to control. Each value represents the mean+SD of three experiments.

Table 5: Superoxide scavenging assay activity of Maramanjal Kudineer Chooranam decoction.

Different concentration of extract	Percentage of Superoxide scavenging activity	
	Maramanjal Kudineer Chooranam decoction	Standard Vitamin-C
25 µl/ml	23.64 ± 1.78	19.32± 2.47
50 µl/ml	41.34±1.69	37.32± 1.39
75 µl/ml	59.32 ± 1.56	56.32 ± 1.89
100 µl/ml	78.32 ± 2.34	73.64 ± 2.34
EC_{50} value	57.32	62.34

[a] Results are expressed as percentage of Superoxide scavenging activity with respect to control. Each value represents the mean+SD of three experiments.

Table 6: Metal chelating activity of Maramanjal Kudineer Chooranam decoction.

Different concentration of extract	Percentage of Metal chelating activity	
	Maramanjal Kudineer Chooranam decoction	Standard Vitamin-C
25 µl/ml	19.32 ±1.74	17.34± 1.63
50 µl/ml	34.23 ± 1.69	31.39 ± 1.78
75 µl/ml	51.98 ± 2.37	46.32± 2.34
100 µl/ml	73.64 ± 1.89	67.32± 1.34
EC_{50} value	64.31	67.23

[a]Results are expressed as percentage of Metal chelating activity with respect to control. Each value represents the mean+SD of three experiments.

CONCLUSIONS

As the literature evidences support the usage of drug in the disease, also raw drug are also easily available , as the phytochemical study ,in vitro assays of Decoction from extracts of "Maramanjal Kudineer chooranam" shows better constitution and effects as anti-cancer activity, and moreover Maramanjal has been known and considered as a natural source for using as antioxidant, and anticancer agents compared to commercially available synthetic drugs it will be a better solution in treatment of Cancer and it can be used widely as Medicine for cancer patients by physician's.

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