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Synthesis, characterisation and pharmacological assessment of nanoparticles of *Tinospora cordifolia* for its cytotoxic activity

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Abstract
The present study was taken up to evaluate the use of *Tinospora cordifolia* stem extract as a reducing agent for silver nanoparticle formation and characterization of the synthesized nanoparticles and assessment of its cytotoxic effect on the HepG2 cancer cell line. Silver nanoparticles were synthesized and characterized by visualizing the colour change, observing for the concentric rings under an optical microscope, analyzing the particle size and measuring the zeta potential. The extract was also phytochemically characterized and subjected to MTT assay to evaluate its cytotoxic activity at three different doses of 200 µg/ml, 400 µg/ml and 600 µg/ml against a negative control and a positive control 5-Flurouracil 5 µg/ml. The synthesized nanoparticles were confirmed by visualizing the colour change from colourless to slight reddish brown colour, observing concentric rings under optical microscope, determining the particle size in the range of 0.4 nm and zeta potential was measured to be -13.4mv with peak area of 100 percentage intensity. Phytochemical analysis revealed the presence of saponin, terpenoids, flavanoids, hydrolysable tannin, glycosides and cardiac glycosides in the aqueous extract. The cytotoxicity assay revealed a dose dependent significant decrease in the percentage inhibition of growth of cells with the highest level of inhibition noticed at the highest dose similar to positive control group. Thus the cytotoxic effect of the extract could be attributed to the presence of the secondary metabolites in them which could get converted into nanoparticles with silver and establishes its potential as a chemotherapeutic option for cancer.

Keywords: *Tinospora cordifolia*, silver nanoparticles, x-ray diffraction, particle analyser, MTT assay, cytotoxic activity

Introduction
Nanotechnology is a fast evolving science involved in the production and utilization of nano-sized particles [1]. Noble metals like silver, gold and platinum exhibit a particularly wide range of material behavior along the atomic to bulk transition [2]. Various nanoparticles can be synthesised from plant extracts namely Silver nanoparticles, Gold nanoparticles, Zinc nanoparticles and Copper oxide nanoparticles. Among these, silver nanoparticles have many advantages due to their stability, good conductivity, antimicrobial activity, eco-friendliness, non-pathogenic nature and cost-effectiveness. The activity of silver nanoparticles depends on various factors such as size and shape, surface chemistry, distribution, particle composition and morphology, capping, agglomeration, etc. [3]. In the recent past, in India, the incidence of cancer is increasing alarmingly with 15 lakh people getting affected every year. The conventional methods employed in the treatment of cancer like surgery, radiation and chemotherapy causes adverse effects like organ dysfunction and radiation induced complications [4]. *Tinospora cordifolia commonly known as guduchi* belongs to the family Menispermaceae and is a genetically diverse, large, deciduous climbing shrub with greenish yellow typical flowers, found at higher altitude [5]. The plant mainly contains biologically active chemical constituents like alkaloids, glycosides, steroids, sesquiterpenoid, aliphatic compound, essential oils, mixture of fatty acids and polysaccharides and possess medicinal properties like anti-diabetic, anti-periodic, anti-spasmodic, anti-inflammatory, anti-arthritic, anti-oxidant, anti-allergic, anti-stress, anti-leprotic, anti-malarial, hepatoprotective, immunomodulatory [6] and anti-proliferative activities [7]. The aim of the present study was to evaluate the use of *Tinospora cordifolia* stem extract as a reducing agent for silver nanoparticle formation and the characterisation of the synthesized nanoparticles and to evaluate its cytotoxic effect on the cancer cell line.

T. cordifolia stem

Materials and Methods

Collection and preparation of *Tinospora cordifolia* stem powder

All the glassware were cleaned and washed with double distilled water to remove any impurities and dried in an oven before use. *Tinospora cordifolia* stems were collected and rinsed thoroughly with distilled water and shade dried for 15 days. The dried stem cuttings were finely powdered and used for preparation of the extract by cold maceration method. 25 g of such powder was mixed with 100 mL of sterile autoclaved distilled water and boiled for 15 minutes. Then the material was filtered through whatman No.1 filter paper and the extract was prepared. (Fig 1). The prepared extract was maintained at 4 ^{o}C for further investigations.

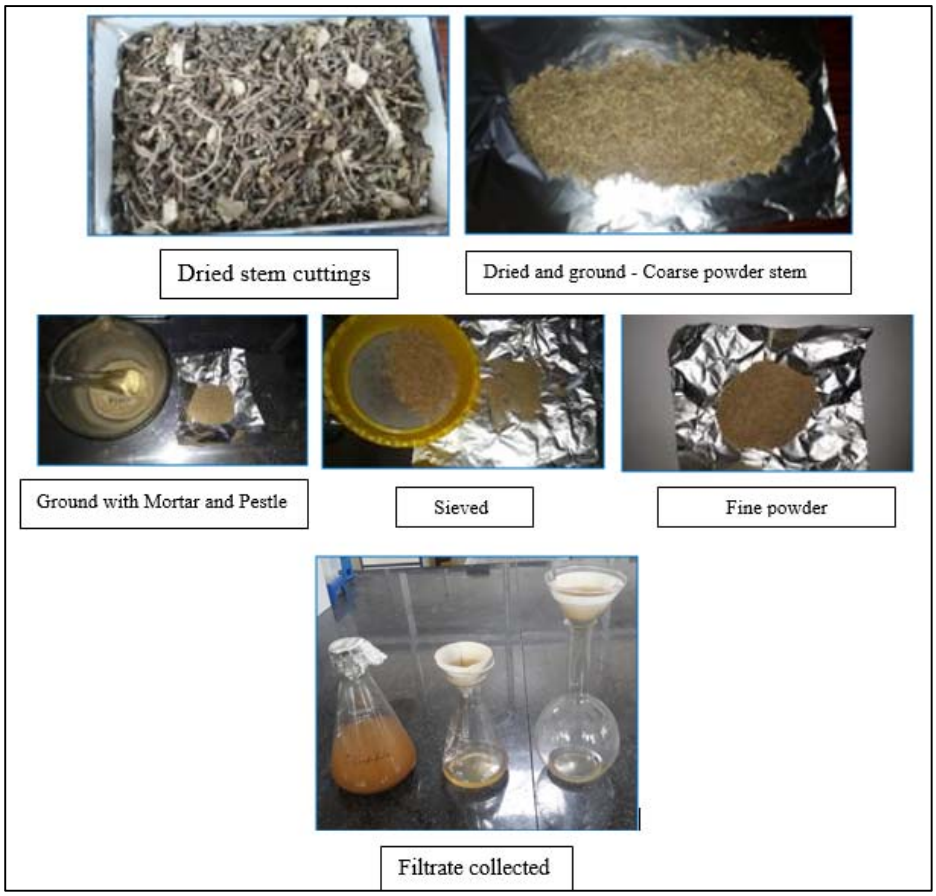

Fig 1: Preparation of the extract

Synthesis of Silver Nanoparticles

Silver nitrate purchased from Sigma Aldrich chemicals was used for the study. The filtrate was used for silver nanoparticle synthesis. 0.1 M $AgNo_3$ was prepared by dissolving 4.25 g $AgNo_3$ in 250 mL double distilled water. 10 mL of the aqueous extract of *Tinospora cordifolia* stem was added to 240 mL of 0.1 M aqueous $AgNo_3$ solution at room temperature for the reduction of silver nanoparticles. The reduction of silver ions takes place within 15 min at room temperature. Slowly the color started changing from colorless to brown and finally reddish brown, indicating the formation of silver nanoparticles.

Characterization of Silver Nanoparticles

The reduction of silver nitrate to pure Ag+ ions using aqueous extract of *Tinospora cordifolia* stem was characterized by using the following techniques.

Colour change

The primary confirmation of the synthesized silver nanoparticles is done by visual basis. The colour change was observed with respect to time.

Analysis by Optical microscope

Optical microscope has been employed for the characterization of the synthesized silver nanoparticles. The particles were visualized with an optical microscope at a magnification of 40X. The sample for visualization were prepared by placing a drop of silver nanoparticles on a slide and observed under the microscope.

Determination of particle size

The dried powders of *Tinospora cordifolia* silver nanaoparticles was dispersed in water to obtain proper

scattering intensity of the nanoparticles. The particle size was determined by Malvern Zeta size analyser.

Determination of Zeta potential

The Zeta potential was measured by using Zeta sizer (Malvern Instruments) having zeta cells, polycarbonate cell with gold plated electrodes and using water as medium for sample preparation. Zeta potential determines the surface potential of silver nanoparticles and it is essential for the characterization of stability of nanoparticles.

Phytochemical Screening

Phytochemical screening of the herbal preparation was carried out using standard chemical methods to identify for the presence of various chemical constituents [8].

***In vitro* Cytotoxicity Analysis**

Cell culture

Human liver carcinoma cell line (HepG2) maintained at the Department of Animal Biotechnology, Madras Veterinary College was used for the study. The cells were cultured as monolayer in Minimum Essential Medium supplemented with 10% Foetal Bovine Serum, 1% glutamine, and 100U/mL penicillin-streptomycin at 37 °C in 5% CO_2 atmosphere. Stocks were maintained in 25cm^2 tissue culture flask.

Cell treatment

The cultured cells were taken after trypsinisation and the cells were resuspended in the medium. With the use of sterile micropipette, the 100µl of the medium along with cell suspension was added in each of the cells of sterile micro titter plate (96 wells). The inoculated plates were kept for incubation about 24hours at 37 °C in 5% CO_2 atmosphere. After incubation, the cells were treated with 200µl of the medium with different concentration of the nano-metallic compounds (600µg, 400 µg and 200 µg) for 48 hours compared with standard anticancer drug i.e. 5-Flurouracil (5µg) and the cytotoxicity was observed by (3-4, 5-dimethyl thiazol- 2yl)- 2,5-di phenyl tetrazolium bromide (MTT) assay. After 48hrs of treatment, the drug was removed, and 200 µl of fresh medium and 50µl of MTT dye was loaded to all the wells and allowed to incubate for 4hr with the late wrapped with aluminium foil. After incubation, the medium was removed and 100 µl of DMSO was added to dissolve the formazan crystals and the cells were read at 570nm using ELISA reader.

Percentage of cell inhibition =100 – [(At-Ab)/Ac-Ab) x 100]

(At-Ab)/Ac-Ab) x100 gives cell survival.

At - Absorbance value of test compound

Ab - Absorbance value of blank

Ac - Absorbance value of control

Statistical Analysis

The data collected on various parameters were subjected to Duncan's test as per the method suggested by Snedecor and cochran (1994).

Results and Discussion

Characterisation of Silver nanoparticles

Visual observation

The colour change in the reaction mixture was recorded which indicated that silver nanoparticles were synthesized by using a green method through reduction of silver nitrate (AgNO3) solution by plant extract. After the addition of aqueous extract of stem of *T.C* to the AgNO3 solution (1mM/L), a change in colour was observed from yellow to dark brown in about 15 minutes. The brown colour indicated the green synthesis of silver nano particles. (Figure 2)

Fig 2. Synthesis of nano particles

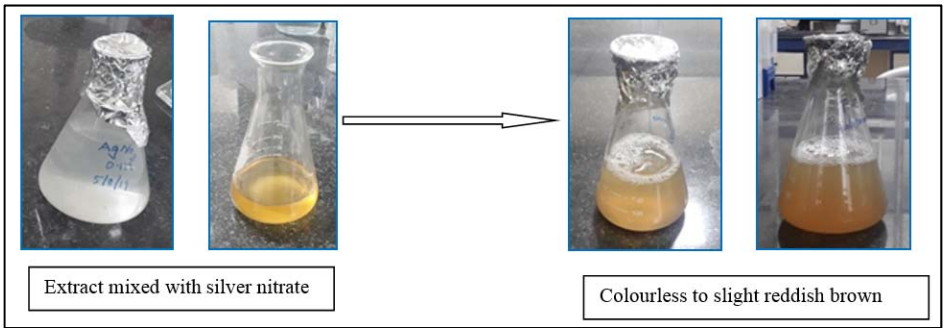

Fig 2: Synthesis of nano particles

Optical microscope

The optical microscope was used to confirm the formation of silver nanoparticles.

It was found that the nanoparticles formed concentric rings of silver nanoparticles. (Figure 3)

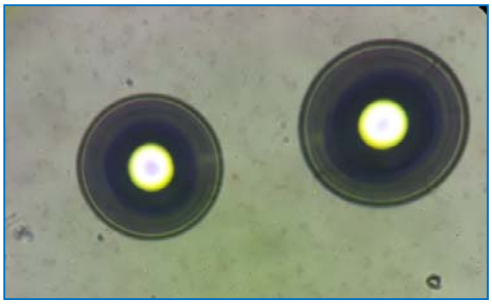

Fig 3: Optical microscopic image of *Tinospora cordifolia* silver nanoparticles

Particle size measurement

The particle size is one of the most important parameter for characterisation of nanoparticles. The average particle size of *T. cordifolia* silver nanoparticles was found to be 0.4 nm (Figure 4) which confirmed the formation of nanoparticles.

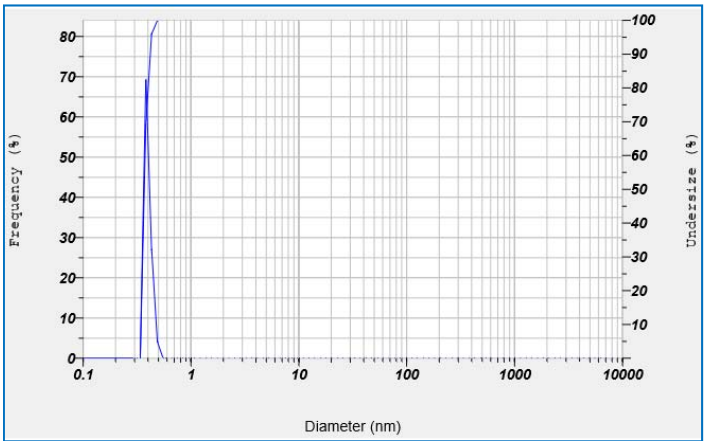

Fig 4: Particle size distribution of *Tinospora cordifolia* silver nanoparticles

Zeta potential measurement:

Zeta potential is a key indicator for determining the stability of aqueous silver nanoparticles. For *T. cordifolia* silver nanoparticles zeta potential measured was found to be -13.4mv with peak area of 100% intensity. These values indicate the full stabilisation of nanoparticles. (Figure 5).

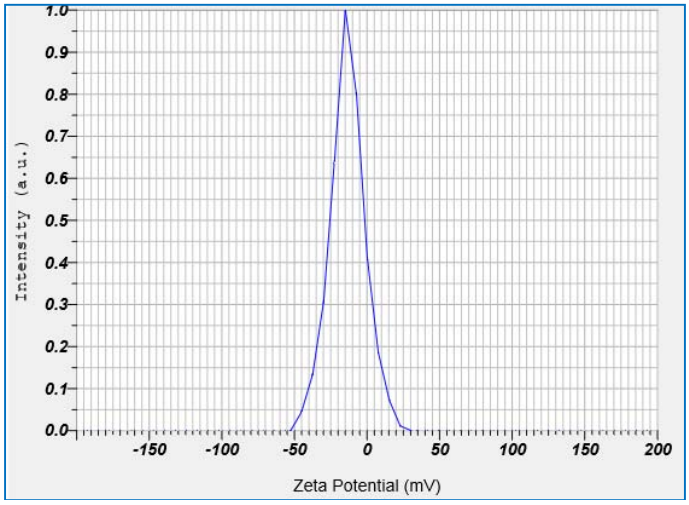

Fig 5: Zeta potential distribution of *Tinospora cordifolia* silver nanoparticles

Phytochemical Analysis

The phytochemical analysis revealed the presence of saponin, terpenoids, flavanoids, hydrolysable tannin, glycosides and cardiac glycosides in the aqueous extract. Table 1 and Figure 6.

Table 1: Phytochemical analysis of the aqueous extract of *Tinospora cordifolia* stem extract

S. No	Phytochemicals	*T. cordifolia*
1	Saponin	+
2	Tannin	_
3	Phenol	_
4	Alkaloid	_
5	Terpenoids	+
6	Flavanoids	+
7	Amino acid and protein	_
8	Carbohydrate	_
9	Phylobatanin	_
10	Volatile oils	_
11	Hydrolysable tannin	+
12	Glycosides	+
13	Cardiac Glycosides	+
14	Vitamin C	_

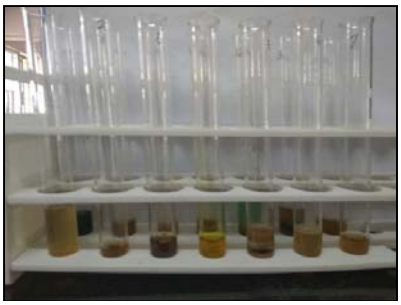

Fig 6: Phytochemical analysis of the aqueous extract of *Tinospora cordifolia* stem extract

***In Vitro* Cytotoxicity Analysis**

The in-vitro cytotoxicity effect of silver nanoparticles studied against HepG 2 cell line at different concentration of 600µg, 400 µg and 200 µg and a positive control is indicated in the Figure 7, Plate 1 and in Table 2.

The cytotoxic effect of the test compound on cell was noticed with detachment of the cells and changes in cell morphology like cell shrinkage, rounding and disintegration in a dose dependent manner similar to the positive control.

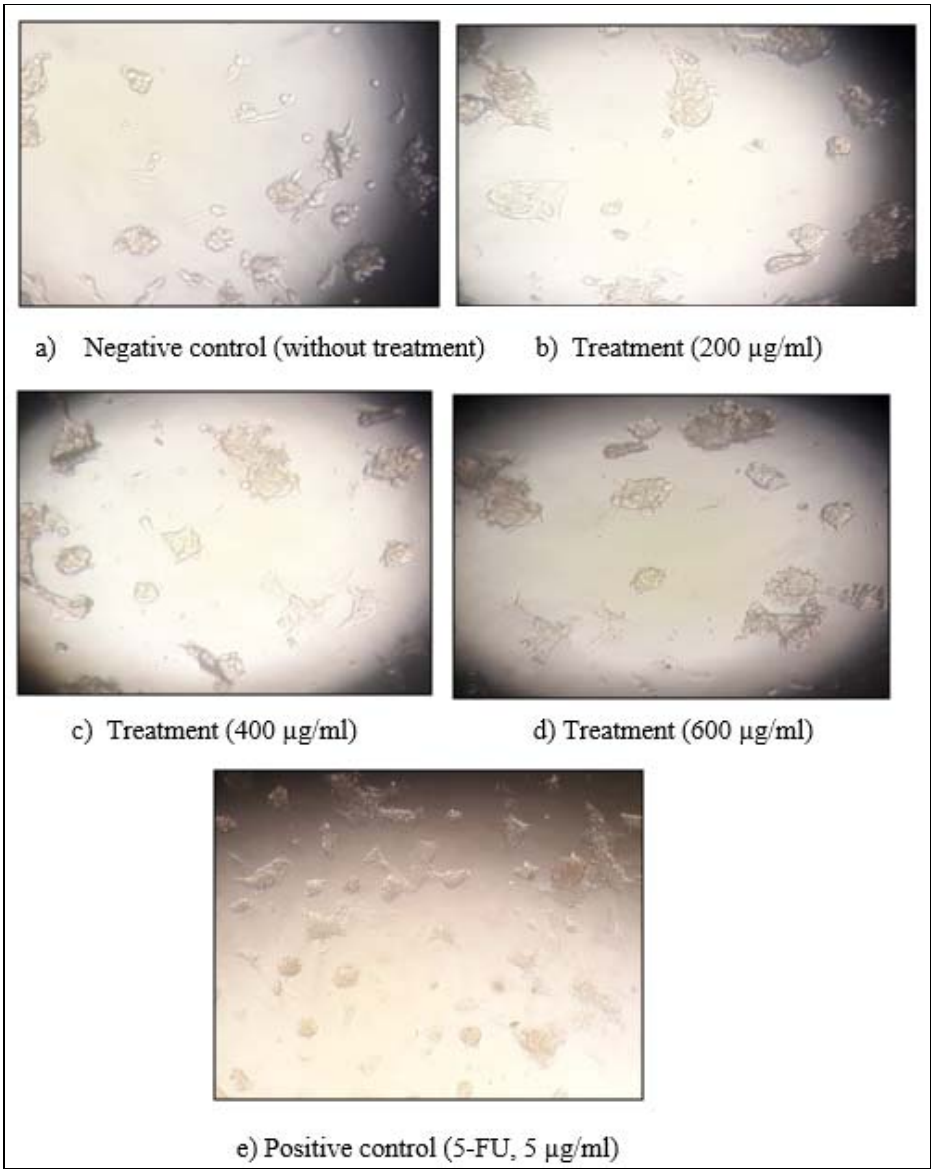

Plate 1: Effect of *Tinospora cordifolia* silver nanoparticles on cell morphology

Table 2: Cytotoxic effect of *Tinospora cordifolia* silver nanoparticles on cell line

Group	Treatment	Percentage of inhibition
I	Negative control	$0^{a} \pm 0$
II	Positive control (5-FU,5 µg/ml)	$45.97^{b} \pm 1.31$
III	*T.C* (200 µg/ml)	$56.43^{c} \pm 0.95$
IV	*T.C* (400 µg/ml)	$67.32^{d} \pm 1.39$
V	*T.C* (600 µg/ml)	$67.79^{d} \pm 1.82$

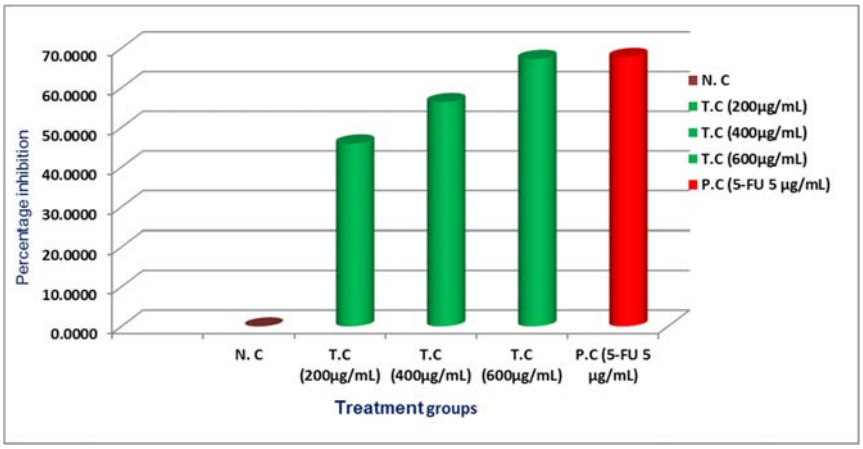

Fig 7: Cytotoxic effect of *Tinospora cordifolia* silver nanoparticles on cell line

There was a significant difference ($p<0.01$) in the percentage of cell inhibition between the groups in a dose dependent manner with the highest level of inhibition noticed at the highest concentration of the test compound similar to the positive control group. The level of inhibition in the highest concentration and the positive control also significantly differed from other groups. The results are in accordance with the findings of [9] who reported 50% cell death in Hep 2G cells on treating with 129.1µg/mL of *Tinosora cordifolia* root extract. Flavonoids form the largest group of natural phenolic compounds and possess excellent free radical scavenging and antioxidant properties. [10]. Also the stem extract of *T.c* was found suitable for the synthesis of silver nanoparticles. In the present study, the cytotoxicity effect of *T.c* is identified with the maximum effect observed at the highest concentration. Silver ions and silver based compounds proves to be toxic to cancer cells because these are highly reactive species. They facilitate the transport of the phytochemical constituent of therapeutic importance like phenolic comounds i.e flavanoids, terpenoids and saponins to the cell surface acting as a drug carrier. More recently, it is shown that silver chelation prevents unwinding of DNA. Silver nanoparticles are composed of silver atoms. They are larger in size than silver ions, which makes them react with more molecules leading to exhibit cytotoxicity [11]. Thus the cytotoxic effect of the extract could be attributed to the presence of the secondary metabolites in them which could get converted into nanoparticles with silver.

Conclusion

The present study demonstrates biosynthesis of silver nanoparticles using stem extract of *Tinospora cordifolia* as a potential reducing and stabilizing agent. Different biomolecules present in the plant extract facilitates the formation of silver nanoparticles. The obtained silver nanoparticles showed significant cytotoxic effect. Thus green synthesis of nanoparticles has several advantages like eco-friendly, low cost and non-toxic and can efficiently be used as a natural alternative to conventional cancer treatments. Therefore, *T. cordifolia* stem extracts have considerable cytotoxic effects on cancer cells and this plant may have a role in preventing human diseases in which free radicals are involved, such as cancer, cardiovascular disease, and premature aging. However, further investigations on the *in vivo* activity are warranted. So, it can be concluded that *T. cordifoila* has the potential to be established as a chemo-preventive option for cancer. But, the molecular mechanism along with the isolation of phyto-constituents responsible for its anti-cancer activity has to be carried out in further studies.

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