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Tridax procumbens: A Herbal Nano Formulation for Cancer Therapy†

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The therapeutic values of commonly available plants are of more interest due to its beneficial pharmacological activities. *Tridax procumbens*, an Asteraceaeous member is a most commonly available weed and pest plant in native topical Americas and few tribal parts of India. This plant has several medicinal properties. Its fresh leaf juice is used to cure wounds, to stop bleeding and also used as a hair tonic. The objective of the present work is to extract the leaves of *T. procumbens* by maceration process using water and to identify the presence of various phyto-chemical constituents. The crude extract was developed into polymer-herbal nanoparticle by solvent evaporation process using polyvinyl pyrrolidine (PVP) and characterized for its size and stability. The crude herbal extract and its nanoparticle formulation were evaluated for antioxidant and antiinflammatory potential through *in vitro* methods and anticancer activity evaluated through MTT assay against HeLa cell lines.

Keywords: *Tridax procumbens*, Maceration, Herbal nanoparticles, MTT assay.

INTRODUCTION

From time immemorial herbs have formed an integral part in medicines and diet for better health care of human society. Records on the usage of herbs as medicines are available since many centuries[1]. These herbal medicines are often prescribed by traditional medicine practitioners to treat various diseases but most of the applications are not supported by empirical data. However, there was a change in the development of herbal medicine with the sudden expansion in the modern medicine inventing a competitive concept of patent pharmaceuticals[2]. There was a debate existing on the use of natural products as for its reliability and immediate recovery. Recently there is an increased interest observed in the rate of herbal medicine usage and usefulness in various diseases. This has lead to a remarkable growth in the development of phytopharmaceuticals[3]. Recently there have been scientific evidences available for the therapeutic applications. Analytical techniques have contributed significantly towards the discovery of active constituents from the herbal plants[4].

For researchers engaged in the development and advancement of novel drug development, the doors of the traditional system of medicine are open. Especially useful in researches focusing bio-availability and half life period for better healthcare. Among the various carriers in novel drug delivery system the most commonly used and biocompatible system is polymeric nanoparticles that could be useful in delivering a wide range of drugs[5]. Nanotechnology is an advanced technique of 21st century, especially in the field of drug delivery. A number of traditional based drug delivery system was developed that possess desirable targeting characteristics and efficient pharmacokinetic and dynamic properties[6].

For the present work, *Tridax procumbens* a very common noxious weed found in the tropical regions of America and Africa with high therapeutic potential is selected. It is an astraceae member and known for its therapeutic properties like antibiotic, antiviral, anti oxidant and insecticidal. This is used in traditional medicines as an anti coagulant, anti fungal and wound healer and also used against ulcer, diarrhoea and dysentery. Most of the reports related with folk medicine in India are usage of its leaves to stop bleeding[7]. In this paper, a comparative study of the crude aqueous extract of *T. procumbens* and its nanoparticle formulations is made and presented. A polymeric nanoparticle formulation is prepared from this herbal weed extract and it was subjected to different *in vitro* investigations such as anticancer, antioxidant and antiinflammatory studies. Qualitative analysis of the phytochemical constituents present in *T. procumbens* was performed to identify bioactive compounds and to check the quality and activity of nanoparticle formulation of the same extract.

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EXPERIMENTAL

Tridax procumbens (Asteraceae family), was collected, identified and authenticated by comparing the specimen deposited at Raphinet Herbarium, St. Joseph College, Trichy, India.

Extraction by cold maceration: About 30 g of the leaves of *Tridax procumbens* was washed and crushed using mortar and pestle by adding few drops of distilled water required for crushing. The cold macerated crude extract was collected separately. The marc was pressed to squeeze out the final traces of the extract and was mixed with the macerated extract. The combined crude leaf extract was allowed to evaporate for 3 h at 50 ºC and finally the dry aqueous extract of the leaf sample was obtained[8].

Extraction by hot maceration: The leaves of *Tridax procumbens* weighing 30 g was dried in hot air oven at 50 ºC for 24 h and then powdered by rotary mixer. To the dry sample, 100 mL of warm water was added and allowed to boil for 20 min at less than 100 ºC. The decoction of the extract was separated by filtration using a muslin cloth and the filtrate was kept for evaporation for 12 h at less than 100 ºC to obtain dry powder extract of the leaves[9, 10].

Preparation of polymeric nanoparticles: The nanoparticles of herbal extract with polymer were obtained by solvent evaporation process. For this nanoparticle extraction an aqueous solution of the extract was prepared by dissolving 0.02 g of dry extract with 10 mL of water and 0.01 g of pluronic F68 which was added as a surfactant. The organic phase containing the polymer was prepared using 0.05 g of poly(vinyl pyrrolidone) with 2 mL of dichloro methane (DCM). The aqueous solution was sonicated for 20 min at 100 KV using a probe sonicator (P250 Vibronics, India), to which the organic solvent was poured in drops. Then the mixture was constantly stirred using a magnetic stirrer for 20 min, until the organic solvent is completely evaporated[11,12]. This process was performed for the extracts obtained by cold and hot maceration to formulate their respective nanoparticles. A blank nanoparticle containing the polymer and surfactant (without extract) was also formulated for comparative studies.

Evaluation of the nanoparticles

Particle size and surface charge analysis: The nanoparticle formulations of both the extracts were analyzed by a zeta analyser (Malverm Nano Series ZS, UK) to verify the particle size based on dynamic light scattering technique and the zeta potential based on charge conductivity principle, to ensure the uniformity of size distribution and the stability of the formulation respectively[13].

Anticancer activity: MTT assay is a colorimetric technique for measuring the viability of the cells. MTT is a water soluble yellow tetrazole compound that enters the cell, where its tetrazolium ring can be cleaved by the mitochondrial enzyme succinate-dehydrogenase present in living cells, which gets reduced to purple coloured insoluble formazan crystals. When the cells are solubilized by an organic solvent, the formazan solubilizes into the cytoplasm of the cell giving a purple colour, which can be measured spectrophotometrically at 570 nm. The intensity of colour is proportional to the number of viable cells present in each well, from which the % growth inhibition can be estimated. This test was performed for the crude extract, nanoparticles of *T. procumbens* and for blank nanoparticles without extract[14].

The 96 well plates were seeded with HeLa cell lines (Cervical cancer cells) and incubated for 24 h. After 24 h the cold macerated extract and the nanoparticles of cold extract were seeded separately in different plates and incubated for 1 h. After this, the plates were removed from the incubator and 10 µL of MTT was added to each well plate and kept for 4 h incubation. Then the supernatant was carefully removed, taking care that the formazan crystals formed were not removed and finally 100 µL of isopropyl alcohol was added to each well. The plates were kept on a shaker until the crystals were dissolved[15]. The absorbance of the dissolved formazan was measured at 570 nm and the percentage growth inhibition was calculated using the formula,

$$\text{Growth inhibition (\%)} = \frac{\text{Control} - \text{Test}}{\text{Control}} \times 100$$

***in vitro* Antiinflammatory bioassay:** 2 mL of varying concentrations of the sample (aqueous crude extract prepared by cold and hot maceration and their respective nanoparticles in separate test tubes) was taken and added to a mixture containing 0.2 mL of egg albumin and 2.8 mL of phosphate buffered saline (PBS, pH 6.4), so that final concentrations of the solution was 30, 60, 120, 240, 500 µg/mL. Distilled water of similar volume was used as control. Then these samples were incubated at 37 ± 2 ºC for 15 min and then heated for 5 min at 70 ºC. After cooling, the absorbance was measured at 660 nm using respective vehicle blank. Diclofenac sodium was used as a reference drug, which was treated similarly with egg albumin and the final concentration of the solutions were measured by its absorbance[16]. The percentage inhibition of protein denaturation was calculated as

$$\text{Inhibition (\%)} = \frac{V_t}{V_c - 1} \times 100$$

where, V_t = absorbance of test sample, V_c = absorbance of control.

Antioxidant activity by ferric reducing assay: Different concentrations of the crude aqueous extract obtained by cold and hot maceration and their nanoparticles (100, 200, 300, 400 and 500 µg/mL) were dissolved in 1 mL of methanol. To each test tube 2.5 mL of phosphate buffer pH 6.6 and 2.5 mL of 1 % potassium ferricyanide were added. These tubes were kept in water bath at 50 ºC for 20 min and then cooled rapidly. The cooled samples were mixed with 2.5 mL of 10 % trichloroacetic acid and 0.5 mL of 0.1 % ferric chloride and this mixture was incubated for 10 min. A Perl's Prussian blue colour formed due to the presence of iron(II)-ferricyanide complex, was measured for the absorbance at 700 nm. The increase in absorbance of the reaction mixtures indicates increased reducing power[17-19].

Phytochemical analysis: Preliminary phytochemical screening was carried out for extracts and their respective nano formulation as per standard texual procedures[20-25].

RESULTS AND DISCUSSION

Effect of extraction process on particle size and zeta potential of nanoparticles: The nanoparticles of the aqueous extract of *T. procumbens* prepared by cold maceration showed narrow particle size distribution with average size observed as 200 nm. The surface charge measured as zeta potential was -17.2 mV which ensured a stable formulation. The nanoparticles of aqueous extract obtained by hot maceration showed wide size distribution pattern, with an average particle size of 500 nm and the zeta potential as -10.8 mV. Comparing the size distribution and charge, the hot macerated extract nanoparticles was found to show poor stability and higher size, compared to the nanoparticles of cold maceration (Figs. 1 and 2).

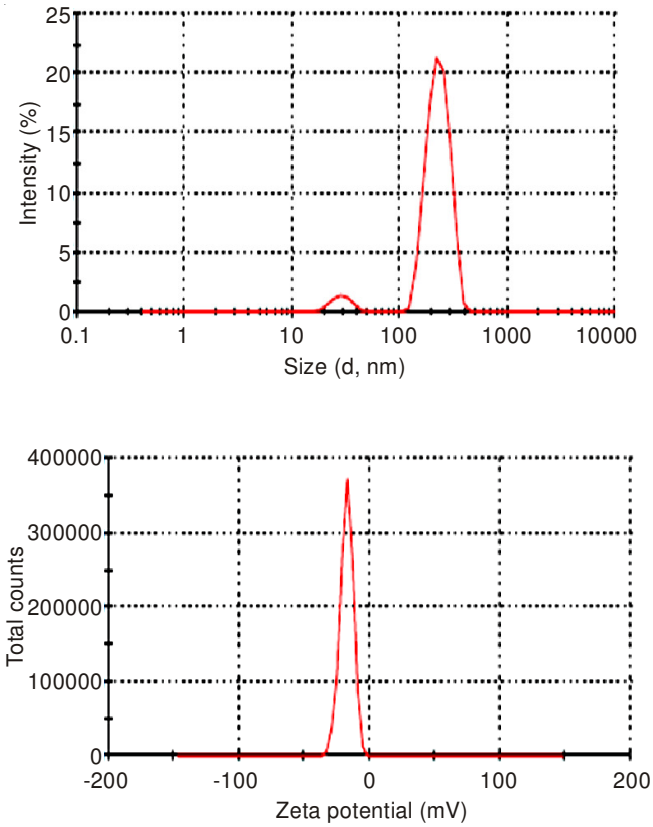

Fig. 1. Size distribution and charge analysis of *T. procumbens* nanoparticles prepared by cold maceration

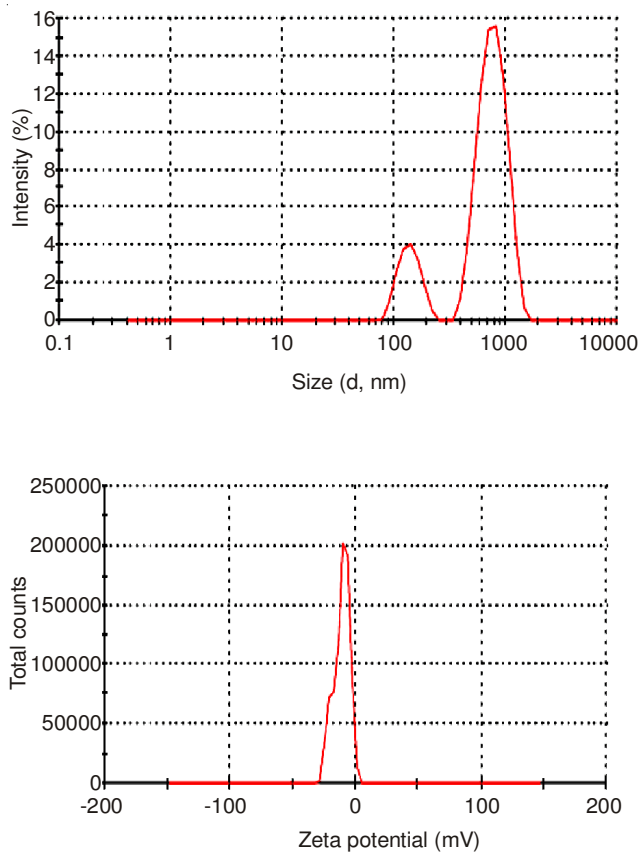

Fig. 2. Size distribution and charge analysis of *T. procumbens* nanoparticles prepared by hot maceration

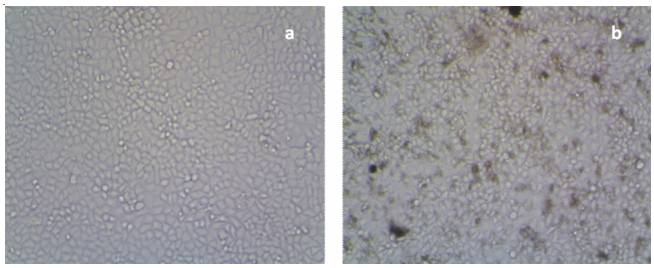

Fig. 3. Percentage cell inhibition of *T. procumbens* extract on HeLa cell lines by MTT assay (a) control (b) 300 µg/mL

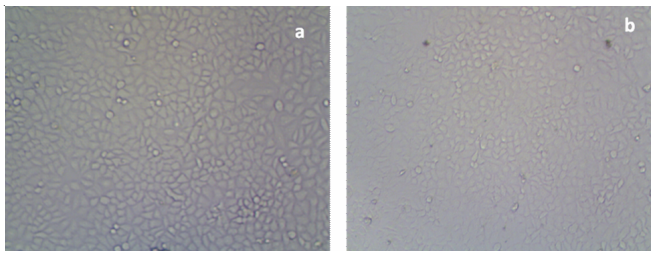

Fig. 4. Percentage cell inhibition of *T. procumbens* nanoparticles on HeLa cell lines by MTT assay (a) Control (b) 50 µg/mL

Anticancer efficacy of nanoparticles: MTT assay results revealed the presence of cell death in the HeLa cell lines after the administration of the *T. procumbens* extract and their nanoparticles, when compared with the control sample without the extract. Fig. 3 shows the comparison of cold extract sample with the control, where the extract concentration at 300 µg/mL showed the maximum % growth inhibition as 21.3 %. Fig. 4 shows the comparison of the nanoparticle of the extract with the control, wherein the maximum concentration of 50 µg/mL showed the % cell inhibition as 22.24 %. The results in Table-1 ensured that the % cell inhibition increased as the concentration of the sample increased and the IC_{50} value was comparatively lesser for the nanoparticle formulation of the extract than the crude extract. Around 20 % growth inhibition was observed at 300 µg/mL concentration of the pure extract, whereas the same effect could be obtained in 50 µg/mL concentration of the nanoparticles. The data thus obtained revealed that the polymeric nanoparticle formed with cold aqueous extract showed a better anticancer activity than the crude aqueous extract. Usually nanoparticles were known to improve bioavailability and these nanoparticles were expected to

penetrate through the ion channels of cells without causing damage to cell membranes suppressing the enzyme and protein expression of the diseased condition. This could be the main reason for the increased anticancer activity observed in nanoparticles[26,27] (Table-1).

TABLE-1
% CELL INHIBITION OF CRUDE EXTRACT AND THE NANOPARTICLES OF *T. procumbens* ON HeLa CELL LINES

Conc. (µg/mL)	TPC	Conc. (µg/mL)	TPNC
18.75	4.23 ± 0.006	3.125	-2.47
37.5	7.67 ± 0.005	6.25	-1.72
75	7.67 ± 0.005	12.5	9.06
150	16.07 ± 0.004	25	16.55
300	21.36 ± 0.004	50	22.24

TPC: *T. procumbens* crude extract by cold maceration, TPNC: *T. procumbens* polymeric nanoparticles of cold macerated extract.

Antioxidant property for *T. procumbens* nanoparticles: The results of antioxidant activity of the crude extracts obtained by hot and cold maceration and their respective nanoparticles are presented in Table-2. The assay was performed for five different concentrations (100, 200, 300, 400, 500 µg/mL) and it was observed that the spectrometric results suggested an increased reducing power as the concentration of extract increased. The data of the crude extracts and their nanoparticles were compared with the standard values obtained from ascorbic acid. The data showed the highest reducing power of ferrous to ferric in the assay[19], depicting the scavenging of the free radicals by the polymeric nanoparticles prepared using the extracts obtained by cold maceration. The polymeric nanoparticle prepared by hot maceration showed higher consumption of the free radicals than the crude aqueous extracts in the lower concentrations, but as the concentration of the nanoparticle extract increased there was a decrease in the antioxidant property. Also, at higher concentration of the crude aqueous extract prepared by cold maceration an increase in the reducing power than the nanoparticles obtained by hot maceration was observed. Standard assay for ascorbic acid was also performed by FRAP method and the results showed that the formulations with extract of *T. procumbens* showed a higher activity. The antioxidant activity in all the *T. procumbens* formulations performed showed an increased activity and this can be due to the presence of flavonoids which was confirmed in the phytochemical analysis done[28].

Antiinflammatory effect of crude extract and nanoparticles: *T. procumbens* showed high protein denaturation effect *in vitro* when egg albumin was used as the protein. *T. procumbens* as crude extract showed a higher antiinflammatory activity when compared to diclofenac sodium that was used as a standard. The comparative evaluation presented in Table-3 depicted a cumulative evaluation of the standard, crude extract and the nanoparticles formulated from both extracts. Higher activity is noticed in the nanoparticle formulations, especially with increasing concentration of the extract. This suggested that the percentage inhibition was higher in the nanoparticle formulation prepared from both the extracts, as compared to the crude aqueous extract samples and the standard.

Phytochemical analysis: Preliminary phytochemical screening was carried out for the crude aqueous extract of *T. procumbens* and the nanoparticles prepared from both cold and hot maceration. The screening was performed separately for each sample and qualitatively tested for the presence of saponins, tannins, flavonoids, steroids, terpenoids and phlobatannins. The presence or absences of these constituents were given in Table-4. The presence of tannins, flavanoids, steroids and terpenoids were confirmed in all the samples, whereas phlobatannins was absent in all the samples. The saponins and terpenoids identified in the crude extract samples were not shown in the formulated nanoparticles which may be due to the encapsulation and interaction of the polymer with the extract during the nanoparticle preparation. The presence of flavonoid showed a high intensity in the colour which was observed visually. This flavonoid is the main reason for its free radicals scavenging property resulting in a high antioxidant property[28]. The presence of tannin in *T. procumbens* can be correlated with antiinflammatory activity as tannin as humic acid possess high antiinflammatory activity[17].

TABLE-2
REDUCING POWER OF *T. procumbens* EXTRACT AND NANOPARTICLES

Concentration (µg/mL)	Ascorbic acid	TPC	TPH	TPNC	TPNH
100	0.034 ± 0.01	0.23 ± 0.002	0.225 ± 0.020	0.316 ± 0.002	0.306 ± 0.001
200	0.106 ± 0.003	0.246 ± 0.002	0.228 ± 0.015	0.353 ± 0.002	0.314 ± 0.003
300	0.12 ± 0.015	0.457 ± 0.004	0.341 ± 0.002	0.491 ± 0.004	0.346 ± 0.001
400	0.24 ± 0.075	0.52 ± 0.005	0.391 ± 0.006	0.594 ± 0.006	0.423 ± 0.002
500	0.38 ± 0.016	0.513 ± 0.001	0.421 ± 0.005	0.606 ± 0.005	0.492 ± 0.006

TPC: *T. procumbens* crude extract of cold maceration, TPH: *T. procumbens* crude extract by hot maceration, TPNC: *T. procumbens* polymeric nanoparticles of cold macerated extract, TPNH: *T. procumbens* polymeric nano particles of hot macerated extract.

TABLE-3
COMPARATIVE ANALYSIS OF *IN VITRO* ANTI INFLAMMATORY ACTIVITY OF CRUDE EXTRACT AND NANOPARTICLES OF *T. procumbens* WITH STANDARD DRUG DICLOFENAC SODIUM

Concentration (µg/mL)	Diclofenac sodium	TPC	TPH	TPNC	TPNH
	Inhibition (%)				
30	6.30 ± 0.08	33.29 ± 0.05	23.39 ± 0.03	518.13 ± 0.04	490.59 ± 0.03
60	15.33 ± 0.09	75.02 ± 0.08	35.35 ± 0.09	672.64 ± 0.02	518.38 ± 0.04
120	34.62 ± 0.09	123.68 ± 0.08	50.23 ± 0.01	745.36 ± 0.02	536.62 ± 0.06
240	76.72 ± 0.03	316.72 ± 0.01	120.3 ± 0.09	836.45 ± 0.07	627.45 ± 0.05
500	123.33 ± 0.02	750.29 ± 0.06	202.5 ± 0.05	1324.59 ± 0.02	818.53 ± 0.01

TABLE-4
QUALITATIVE ANALYSIS OF PHYTOCHEMICAL CONSTITUENTS IN *T. procumbens*

Formulation	Saponins	Tannins	Phlobatannins	Flavonoids	Steroids	Terpenoids
TPC	+	+	–	+	+	+
TPH	+	+	–	+	+	+
TPNC	–	+	–	+	+	–
TPNH	–	+	–	+	+	–

+: Represents presence, –: Represents absence.

Conclusion

Tridax procumbens a commonly available weed known for its high therapeutic values in folk medicine is gaining interest in recent times. The alcoholic or hydro-alcoholic extracts of *T. procumbens* can be commonly obtained and these alcoholic extracts showed a high antibacterial and anticancer activity. Similarly the silver nanoparticles of *T. procumbens* also showed different biopharmaceutical activities *in vitro.* In the present work the crude aqueous extract of *T. procumbens* and polymeric nanoparticles showed a greater activity than the commonly used commercial standard drugs. The nanoparticles formulation exhibited a high anticancer, antioxidant and antiinflammatory activity. The presence of various chemical constituents can be attributed to different activities which make the nanoparticles of *T. procumbens* as an effective herbal formulation. Inventions of phyto-pharmaceuticals using nanotechnology can lead to an improved therapeutic agents with enhanced potentials and can contribute towards the healthcare of human society.

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