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Entrapment of Stem Extracts of *Cissus quadrangularis* into Biodegradable Nanoparticles and its Effectiveness for Diverse Therapeutic Activities†

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Nature has been serving as the source of medicinal plants for several decades but presently a tremendous increase in the field of ethnobotany has emerged, dealing with the therapeutic value of traditional plants. New materialization techniques have paved the way to develop nanoparticles of ethnomedicinal plant *Cissus quadrangularis* Linn commonly called as "Pirandai" in Tamil. *C. quadrangularis* L is known to possess several pharmacological activities. Plant is extracted with petroleum ether and ethanol and its active constituents were identified through GC-MS analysis. The nanoparticles were developed by incorporating the extracts with polyvinyl pyrrolidone (PVP) as encapsulating agent and optimized for various parameters to characterize and stabilize the formulation. The crude ethanolic extract and its polyvinyl pyrrolidone-nanoparticle formulation were analyzed for their anticancer efficacy against MCF-7 (breast cancer) cell lines. Antiinflammatory and antioxidant activities were studied for the crude extracts and respective nanoparticles.

Keywords: MCF-7 cell lines, *Cissus quadrangularis* L, Anticancer, Antiinflammatory, Antioxidant activity.

INTRODUCTION

Application of nanotechnology in the field of pharmaceutical industry and research has reached the peak due to its various advantages in drug delivery such as increased bioavailability and enhanced solubility, sustained release, targeted delivery at specific site and reduced toxicity. Various nano systems are in existence, among which the polymeric nanoparticles are significant due to certain inherent properties like biocompatibility and biodegradability. Besides, they usually form colloidal dispersion system with particles ranging from 100-1000 nm in size. Polymeric nanoparticles of a drug are designed using either synthetic or natural polymers. These nanoparticles are broadly classified as reservoir or vesicular systems (nanocapsules) and matrix systems (nanospheres). In nanocapsules, the drug is narrowed to a specific cavity and coated by a polymer, whereas in case of nanospheres the drug is dispersed throughout the polymeric matrix. Implementation of nanotechnology using polymers in the development of herbal extract nanoparticles is a newer approach.

Cissus quadrangularis L a perennial plant belonging to the family *Vitaceae* is rich in many active constituents which have been proved to act against various diseases and disorders[1-4]. In this work polymeric nanoparticles of *Cissus quadrangularis* extract are produced using synthetic polymer polyvinyl pyrrolidone (PVP) and Pluronic F68 as surfactant. The different extracts of the plant parts of *Cissus quadrangularis* were formulated into nanoparticles and characterized for their physico-chemical stability and various *in vitro* activities[5,6].

EXPERIMENTAL

The whole plant of *Cissus quadrangularis* were collected from Salem district, Tamil Nadu, India. *C. quadrangularis* stem was cut[7] and it was authenticated by comparing the specimen deposited as Raphinet herbarium, Trichy.

Extraction of crude isolates: Stem of *C. quadrangularis* were cut into small pieces, dried in hot air oven at 45 ºC for 48 h and powdered mechanically. Powdered stem was exhaustively extracted using 95 % ethanol (Hayman Limited, England) and petroleum (S D Fine-Chem Limited, Mumbai) ether using Soxhlet apparatus for 72 h. The extract was concentrated at 45 ºC[8-10].

Formulation of polymeric nanoparticles of herbal extract: Polymeric nanoparticles of the herbal extract were prepared by solvent evaporation technique, using polyvinyl pyrrolidone as the polymer for encapsulation of extract. 50 mg of polyvinyl pyrrolidone (Loba Chemie Pvt. Ltd, India) was dissolved in 5 mL of dichloromethane (DCM) (SD Fine-Chem

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Ltd., Mumbai) and sonicated for 10 min to break the particles and then to the dissolved solution 10 mg of extract was added and again subjected to sonication for 5 min. The above mixture acts as the organic phase, which was dispersed drop wise to the aqueous phase containing 1 % Pluronic F68 (Sigma Aldrich, Mumbai) dissolved in 10 mL of distilled water, using probe sonicator for 0.5 h. Subsequently the mixture was homogenized using mechanical stirrer (Elektrocraft India Pvt. Ltd, Mumbai) for 0.5 h followed by stirring using a magnetic stirrer and solvent is evaporated totally. The final dispersion was centrifuged at 3500 rpm for 10 min to separate the unentrapped macro molecules. The supernatant dispersion containing uniform nanoparticles was stored at room temperature for further studies. Similarly nano particles with different concentration of polymer (100, 150 mg) were formulated and optimized[11,12].

Gas chromatography-mass spectrometry (GC-MS) analysis: GC-MS analysis of ethanolic extract was carried out on a GC clarus 500 Perkin Elmer system interfaced to a mass spectrometer employing the following conditions 30 µm × 250 µm film thickness, composed of 5 % phenyl 95 % dimethylpolysiloxane, helium (99.9 % purity) was used as a carrier gas at a constant flow rate of 1 mL/min and an injection volume of 10 µL was employed, with injector and ion source temperature as 270 and 200 ºC, respectively. The oven temperature was programmed from 50 ºC @ 8 ºC/ min to 250 ºC (5 min). Mass spectra were taken at 70 eV (electron ionization technique) @ a scan internal of 0.2 s and resulted fragments scanned from 40-600 Da. The spectrum of separated components was compared with the spectrum of NIST library database[8].

Entrapment efficiency: The extracts were dissolved in methanol, suitably diluted and scanned for its specific UV-visible range of absorbance maxima, which was found to be 410 nm. The absorbance of the samples was measured at 410 nm for different concentrated solutions and a standard calibration curve was plotted using concentration against absorbance.

The nanoparticle suspension formulated with the extract and polymer was ultracentrifuged at 18,000 rpm for 0.5 h. The supernatant solution was diluted suitably to measure the absorbance, from which the concentration of drug in supernatant was calculated using the standard calibration data. The entrapment efficiency of the extract in the polymeric nanoparticles was calculated using the formula[13].

$$\text{Entrapment efficiency (\%)} = \frac{\text{Total drug content–Drug content in supernatant}}{\text{Total drug content}} \times 100$$

Particle size distribution and surface charge analysis: The formulated nanoparticles were analysed for particle size and surface charge using zeta sizer (Malvern Nano Series ZS, UK). The particle size and its distribution and the zeta potential of the nano suspension was measured to ensure the monodispersity and also to predict their stability[14].

Anticancer activity: MTT cell proliferation assay was performed for ethanol extracted sample to estimate the cytotoxicity of crude extract and the formulated polymeric herbal nanoparticles. This was analyzed through a calorimetric method, wherein the intensity of colour produced due to conversion of MTT (yellow colour) into formazan (purple colour) by the mitochondrial enzymes of living cells was measured as the tool to assess the viability of the cells. The 96 well plates was seeded with MCF7 cell lines and incubated for 24 h.

The ethanolic crude extract and its nanoparticles were added separately to the seeded plates and incubated for 1 h. Once the incubation was over, the plates were removed from the incubator and 10 µL of MTT was added to each well plate and again kept for 4 h incubation. The supernatant sample was carefully separated with care that, the formazan crystals formed are not been removed. Finally 100 µL of isopropyl alcohol was added to each well and the plates were kept on a shaker until the crystals were completely dissolved. The absorbance of the formed colour was measured at 570 nm. Percentage growth inhibition was calculated using the formula[15,16].

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

Phytochemical analysis: Qualitative analysis of phytochemical compounds such as saponins, tannins, terpenoids, phlobatannins, flavonoids was tested for their presence in both ethanol and petroleum ether extracts and their respective nano particles[8,17].

Reducing power assay: With ascorbic acid as standard the reducing power of the crude herbal extract and its polymeric nanoparticles were measured. Different concentrations (100, 200, 300, 400, 500 µg/mL) of the plant extract and the nanoparticle formulation was mixed with 2.5 mL of phosphate buffer and 2.5 mL of potassium ferricyanide and incubated at 50 ºC for 20 min. Samples were centrifuged at 10,000 rpm for 2 min. When the formation of the protein precipitation was observed, 2.5 mL from supernatant was treated with 0.5 mL of freshly prepared ferric chloride solution and 2.5 mL of distilled water were added and observed[18,19] for the colour change and the intensity of the absorbance was measured at 680 nm.

***in vitro* Antiinflammatory activity:** A mixture of 5 mL consisting of 0.2 mL of egg albumin, 2.8 mL of phosphate buffer saline (PBS, pH 6.4) and 2 mL of varying concentrations of crude extracts or their nanoparticles was prepared and final concentrations were 40, 80, 160, 320, 640 µg/mL. The same volume of double-distilled water served as control. The above mixtures were incubated at 37 ± 2 °C in an incubator, and then the mixture was heated at 70 °C for 5 min. After cooling the test tubes, the absorbance was measured at 660 nm using the blank. Diclofenac sodium was used as reference drug which were prepared at the concentrations of 78.125, 156.25, 312.5, 625, 1250, 2500 µg/mL, whose absorbance was also measured similarly as the samples. The percentage of inhibition of protein denaturation was calculated by using the following formula[20]:

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

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

RESULTS AND DISCUSSION

Particle size and zeta potential of formulated nanoparticles: Size of polymeric nanoparticles generally lies between 100-1000 nm. The average size of the synthesized nanoparticles of ethanol and petroleum ether extract was found to be within this range. Surface charge plays a major role for a nano particle to be stable, zeta potential of the nano particle acts as the standard criteria to determine the stability of the synthesized nano particle (Fig. 1a). Zeta potential greater than +25 mV and less than -25 mV correlates to a greater stability of the nanoparticle [20] (Fig. 1b). Values, given in Table-1 illustrate that the synthesized nanoparticles were of nanosize and particles were stable.

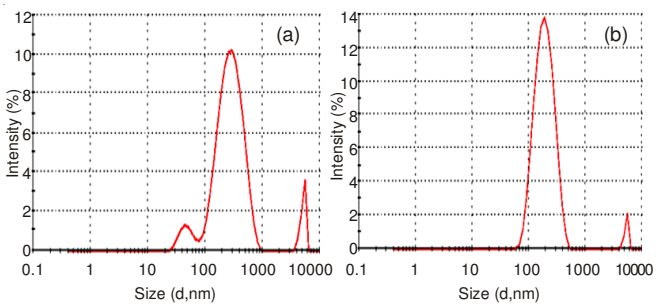

Fig. 1. a: Particle size distribution of (a) Ethanol extract nanoparticles (b) petroleum ether extracts nanoparticles

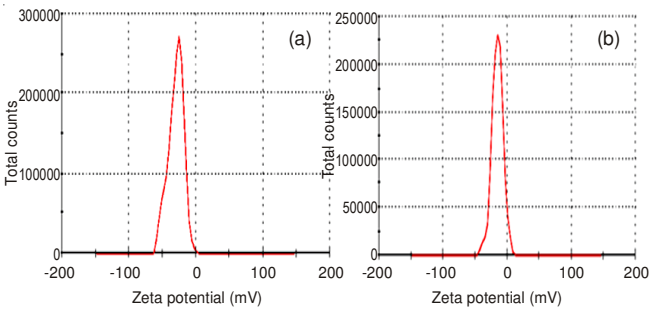

Fig. 1. b: Zeta potential of (a) Ethanol extract nanoparticles (b) petroleum ether extract nanoparticles

TABLE-1
PARTICLE SIZE AND ZETA POTENTIAL OF THE NANOPARTICLES

Sample	Average particle size (nm)	Zeta potential (mv)
Ethanol extract 50 mg	256.9	-29.6
Ethanol extract 100 mg	208.6	-13.3
Ethanol extract 150 mg	155.4	-16.6
Pet-ether extract 50 mg	196.7	-15
Pet-ether extract 100 mg	116.9	-12.4
Pet-ether extract 150 mg	380.8	-10.9

Role of nanoparticles in anticancer activity: The anticancer activity of the crude ethanol extract and nanoparticles were compared using MCF7 cell lines. % cell inhibition showed a gradual increase in value for increased concentration of crude extract with 20.85 % cell inhibition was observed at 18.75 µg of concentration and at a higher concentration of 300 µg % cell inhibition observed was 83.23. For ethanol nano particles 2.79 % cell inhibition observed at a dose level of 12.5 µg and at higher concentration of 100 µg % cell inhibition observed was 36.04. IC_{50} value for ethanol crude and its nano particle were 58.25 and 151.6 µg/mL, respectively (Fig. 2).

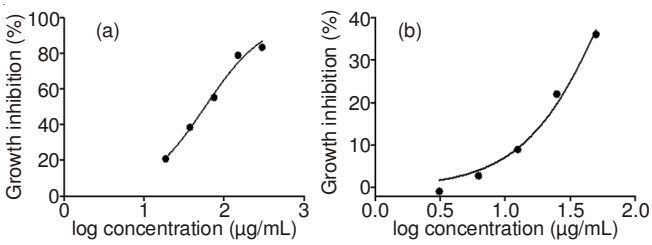

Fig. 2. % cell inhibition of ethanolic extract (a) crude sample (b) nanoparticles

Antioxidant activity: Antioxidant activity was evaluated through ferric reducing antioxidant power assay (FRAP) method which depicted that, the nanoparticles obtained from ethanol extract possess a greater antioxidant activity compared to its crude extract. The petroleum ether crude extract and its nanoparticles revealed almost same activity at different concentrations as presented in Table-2. The absorbance value increases with an increase in the concentration. These absorbance values are compared with the standard (ascorbic acid)[21].

Antiinflammatory activity: Antiinflammatory activity studied by *in vitro* method for different concentration of crude extract and its respective nanoparticles were compared and percentage inhibition was listed in Tables 3a and 3b. The results clearly depicted that the nanoparticles possessed greater antiinflammatory activity at lower concentration whereas the crude extract sample showed better activity only at higher concentration. The crude extracts of ethanol and petroleum ether had greater activity compared to the standard reference drug diclofenac sodium[18,19].

Entrapment efficiency: Amount of extract entrapped in the polymeric nanoparticle determined using UV-visible spectrophotometer which showed that petroleum ether extract nanoparticle had greater entrapment capacity compared to ethanol extract. The entrapment efficiency of ethanol and petroleum ether extract in their respective nanoparticles were 80.91 and 88.34 %, respectively[14] (Fig. 3).

TABLE-2
ANTIOXIDANT ACTIVITY BY FRAP METHOD

Concentration (µg/mL)	Ascorbic acid	Ethanol extract crude	Ethanol extract nanoparticle	Pet-ether extract crude	Pet-ether extract nanoparticle
100	0.034 ± 0.01	0.102 ± 0.05	0.115 ± 0.016	0.108 ± 0.02	0.105 ± 0.01
200	0.106 ± 0.003	0.143 ± 0.02	0.147 ± 0.010	0.134 ± 0.03	0.134 ± 0.01
300	0.12 ± 0.015	0.151 ± 0.01	0.159 ± 0.023	0.140 ± 0.02	0.140 ± 0.02
400	0.24 ± 0.075	0.160 ± 0.02	0.169 ± 0.018	0.161 ± 0.01	0.161 ± 0.02
500	0.38 ± 0.016	0.166 ± 0.01	0.173 ± 0.020	0.162 ± 0.01	0.162 ± 0.01

TABLE-3a
ANTI-INFLAMMATORY ACTIVITY OF DIFFERENT CONCENTRATION OF NANOPARTICLES AND CRUDE EXTRACTS

Conc. (µg/mL)	Inhibition (%)			
	Pet-ether extract nanoparticles	Ethanol extract nanoparticles	Pet-ether extract crude	Ethanol extract crude
40	472.727 ± 0.12	590.909 ± 0.21	33.33 ± 0.32	100.00 ± 0.21
80	509.091 ± 0.11	627.273 ± 0.23	200.66 ± 0.21	150.00 ± 0.28
160	627.273 ± 0.21	781.818 ± 0.35	383.33 ± 0.24	283.33 ± 0.19
320	745.455 ± 0.51	1063.636 ± 0.17	832.43 ± 0.28	1750.0 ± 0.18
640	1072.727 ± 0.31	1690.909 ± 0.11	1200.0 ± 0.11	5083.33 ± 0.29

TABLE-3b
ANTI-INFLAMMATORY ACTIVITY OF DIFFERENT CONCENTRATION OF REFERENCE DRUG DICLOFENAC SODIUM

S. No.	Concentration (µg/mL)	Inhibition (%)
1	78.125	12.5 ± 0.12
2	156.25	12.5 ± 0.133
3	312.5	25 ± 0.271
4	625	50 ± 0.165
5	1250	212.5 ± 0.178
6	2500	812.5 ± 0.199

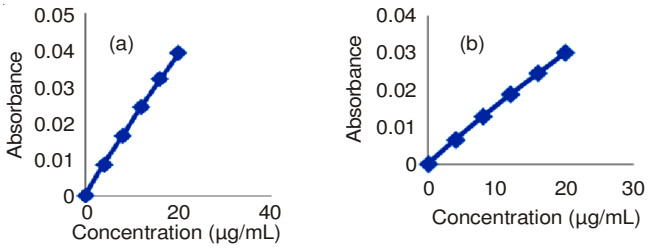

Fig. 3. Calibration curve for (a) petroleum ether and (b) ethanol extract by UV-visible spctrophotomer

Identification of phytochemical constituents by GC-MS: The phytosterols of the plant has been evaluated and validated by simple isocratic chromatographic (HPLC) method[22]. The active compounds of ethanolic extract were identified qualitatively by GC-MS and around 30 compounds were detected, that may be responsible for the activities possessed by the crude extracts and their nanoparticles. The compounds identified with greater percentage peak area in the GC-MS spectra are listed in Table-4. The other compounds found at relatively lower percentage were butylated hydroxytoluene, 2(4*H*)-benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-, dodecanoic acid, benzeneacetic acid, α-methyl-4-(2-methyl-propyl)-, 8,10-undecadiene-3,7-dione, 4-(acetyloxy)-6,6,10-trimethyl-, (E)-, 3-octadecene, (E)-, tetradecanoic acid, 3,7,11,15-tetramethyl- 2-hexadecen-1-ol, 2-pentadecanone, 6,10,14-trimethyl-, 1,2-benzenedicarboxylic acid, *bis*(2-methylpropyl) ester, 5,9,13-pentadecatrien-2-one, 6,10,14-trimethyl-, octadecanoic acid, linoleic acid, methyl ester, stearic acid, heptadecanoic acid, 15-methyl-, ethyl ester, docosanoic acid, ethyl ester, 2R-acetoxymethyl -1,3,5-trimethyl-4c-(3-methyl-2-buten-1-yl)-1c-cyclohexanol, triphenyl phosphate, 1,2-benzenedicarboxylic acid and di-isooctyl ester (Fig. 4).

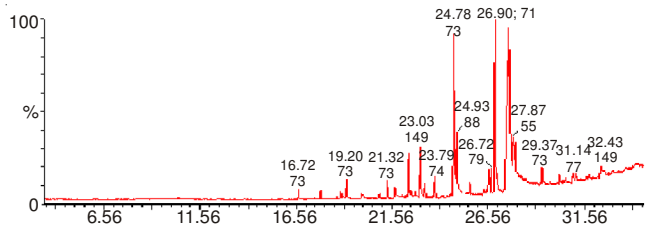

Fig. 4. Identified compounds in GC-MS analysis

TABLE-4
COMPOUNDS WITH GREATER PERCENTAGE PEAK AREA IN GC-MS ANALYSIS

Compound name	m.f.	m.w.	Peak area (%)
Palmitic acid	$C_{16}H_{32}O_2$	256	24.5628
Phytol	$C_{20}H_{40}O$	296	17.7208
Linolenic acid, methyl ester	$C_{19}H_{32}O_2$	292	11.4208
Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	284	11.3404
1,2-Benzene dicarboxylic acid, *bis*(2-methylpropyl) ester	$C_{16}H_{22}O_4$	278	5.3633

Qualitative analysis of phytochemical compounds: Presence of phytochemical constituents[23] in both the extracts are listed in Table-5. Compounds in the crude extract were also present in the nano-formulation prepared.

Conclusion

Nano-formulation using herbal extract was studied to identify whether polymer encapsulated extract shows higher activity than crude extract. A comparative study was made of crude and the nano formulation of both ethanol and petroleum ether extract for their potential activities which depicted a positive and encouraging result. Nano-formulation showed a higher activity. Besides present study revealed that explicitly ethanol extract possess higher activity than petroleum ether extract.

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TABLE-5
QUALITATIVE ANALYSIS OF *Cissus quadrangularis* LINN

Extract	Tannins	Phlobatannins	Saponins	Flavonoids	Steroids	Terpenoids
Ethanol extract crude	+	–	+	+	+	+
Pet-ether extract crude	+	+	+	+	+	+
Ethanol extract nanoparticles	+	–	+	+	+	+
Pet-ether extract nanoparticles	+	–	+	+	+	+

+: Signifies presence; –: signifies absence.

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