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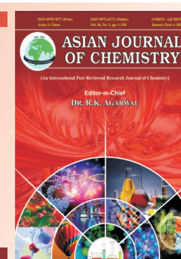

Proteomics and its Related Biological Activity of *Jatropha tanjorensis* Ellis & Saroja: An Ethnomedicinal Plant†

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Traditionally *Jatropha tanjorensis* is being used as an alternative medicine and the phytochemical investigation of this plant has proved that the leaves contain antioxidant, anti-hyperglycaemic and antiinflammatory bio molecules. The crude protein extract was analyzed by non-reducing native-PAGE, which showed three prominent protein bands with molecular weight of about 78.6, 67.8 and 43.3 KDa. Further, 2D studies and mass spectrometric (MS) analysis were done separately. Protein separations were examined using pH 3-10 non-linear IEF strip. Totally 89 protein spots were detected differing in their *p*I values, suggesting a possible difference in their electrostatic charge. In regard to mass spectrometric analysis coupled with in-gel digestions were performed for 5 major protein spots using trypsin and were identified through peptide mass fingerprint of selected peptides in UHPLC-ESI-Quad-TOF and MALDI-TOF mass spectrometer. Proteomic approach lead to the identification of major proteins from *J. tanjorensis*. Among various proteins, redox enzymes and DNA binding proteins were found to be most abundantly extracted proteins. These classes of proteins are known for their antioxidant, antimicrobial and anticancer activities and hence the extracted proteins were evaluated for their biological activities with a view to develop a novel therapeutic agent. The proteins extract revealed potent antioxidant and antimicrobial activities, when compared to standards. It showed IC_{50} value as 173 µg/mL for DPPH scavenging, LPO inhibition (226.2 µg/mL) and hydroxyl ion reducing assays (269.5 µg/mL). In antimicrobial studies it was effective against both gram positive and negative bacteria's such as *S. aureus* MTCC 96, *S. aureus* MTCC EI, *P. aeruginosa* MTCC 741, *P. aeruginosa* MTCC EI, *B. subtilis* MTCC 441 and *E. coli* MTCC 723 with MIC values ranging from 7.8-15.6 µg/mL which are comparable to MIC values of standard antibiotics. *Jatropha tanjorensis* protein extract has also shown potent cytotoxicity against *Ehrlich ascites carcinoma* (EAC) with an IC_{50} of 49.9 µg/mL. Present study scientifically proves the traditional usage of *Jatropha tanjorensis* as a health tonic and for the management of microbial infections.

Keywords: *Jatropha tanjorensis*, MS, 2DE, Antioxidant, MIC, *Ehrlich ascites carcinoma*.

INTRODUCTION

Jatropha tanjorensis (JT) commonly known as "Thanjavur kattamani" is used in Ethnomedicine for the treatment of hypertension, diabetes and as an antiseptic agent. Scientific advancements have lead to the exploration of many such plants chemically for its constituents and their impact in treating several types of diseases. As little attention has been devoted to understand the functional role of ethnomedicinally important plant proteins in relation to its therapeutic use and considering the potential advantage of using plant proteins as drugs (due to non-toxic nature), a through scientific research is attempted. Proteome analysis deals with determination of biological roles and functions of identified proteins[1]. However, the usage of *Jatropha tanjorensis* leaves has been scientifically proven for its therapeutic usage[2] and till now, protein analysis of *Jatropha tanjorensis* leaves have not yet been explored. In this study, proteins from *Jatropha tanjorensis* leaves were extracted, considering that this plant contains rich oil content. Furthermore, besides its high fat content in the leaves, its phenolic contents were also high[3] which tend to obstruct the protein separation process[4]. In order to acquire a good quality and quantity of protein from *Jatropha tanjorensis*, acetone precipitation was performed during protein extraction. Proteomic approaches have brought huge possibilities to evaluate different functions of these proteins in various disease conditions. We investigated the proteome of *Jatropha tanjorensis* and its biological activity using antioxidant and antimicrobial assays. Further to this, cytotoxic assay was also performed on *Ehrlich ascites carcinoma* cells. Proteomic analyses of *Jatropha tanjorensis* leaves revealed the presence of biologically active proteins, most of which correspond to redox metabolism, storage

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proteins, carbohydrate metabolism and stress-related proteins. In this context, preliminary attempts have been made to investigate the proteome of *J. tanjorensis* using advanced proteomic tools to substantiate its ethnomedical claims and explore their possible potential implication in drug development.

EXPERIMENTAL

Jatropha tanjorensis fresh leaves were sourced from different locations in and around SASTRA University, Thanjavur during the month of October 2011 and identified and authenticated by comparing with the Herbarium samples deposited at Raphinet Herbarium, St. Joseph's College, Trichy, Tamil Nadu, India.

Protein extraction: Fresh plant leaves (10 g) were defatted with petroleum ether 60-80 ºC for 4 h and then dried, after which the plant material was homogenized with minimum amount of 1x PBS in chill condition. This was filtered through pre-chilled filter paper and the collected homogenate was cleared off debris by centrifugation for 15 min at 12,000 g at 4 ºC. The supernatant obtained was concentrated using 70 % acetone (protein precipitation). Subsequently, it was dialyzed against cold MilliQ water and kept for lyophilization.

2DE analysis: The protein samples containing 700 µg total/ppt proteins, were resuspended as per the Hoefer lysis buffer (8 M urea, 20 mM DTT, 2 % CHAPS, 1 % ampholytes giving pH 3-10 gradient) and was loaded onto IPG strip trough (24 cm), pH 3-10 non-linear gradient IPG strips (Serva) and was rehydrated at room temperature for 6 h. This was then subjected to IEF in a Hoefer IEF 100 system according to the manufacturer's instruction. IEF configurations are as follows: 18 h at 50 V, 1 h at 1000 V gradient, 1 h at 10000 V gradient, 45000 Vh followed by 1000 V for 1 h constant. After IEF, these strips were equilibrated in an equilibration solution I (6 M urea, 75 mM Tris at pH 8.8, 2 % SDS, 30 % glycerol, 0.002 % bromophenol blue) containing 1 % DTT for 15 min followed by addition of equilibration solution II (3 % iodoacetamide) and incubated for another 15 min. Proteins were further separated in the second dimension through SDS polyacrylamide gels (15 %) at 150 V constant following Laemmli (1970) method[5]. Electrophoresis process was made using 15 % SDS-PAGE gel run at a constant voltage of 150 V. The gel was stained with silver staining solution and the gel image was captured using ChemiDoc XRS imaging system (Bio-Rad laboratories) and protein spots were identified using quantity one software (Bio-Rad laboratories).

In-gel digestion: In-gel digestion was carried out according to a method described by Shen *et al.*[6]. Briefly, each major protein spots were excised from the gel and washed with 25 % v/v methanol and 7 % v/v acetic acid solution until clear background was obtained this was destained using 100 mL of 50 mM NH_4HCO_3 (50 % v/v methanol) for 1 h at room temperature. 10 mM DTT in 100 mM NH_4HCO_3 was added to each protein gel slices for reduction for 1 h and were incubated in 40 mM iodoacetamide and 100 mM NH_4HCO_3 solution for 0.5 h at room temperature. Each gel slice was minced and rehydrated in 100 mM NH_4HCO_3 containing 5 ng trypsin (trypsin, modified, sequencing grade) overnight at 37 ºC. Following trypsin digestion, each protein spot peptides were collected and were extracted with 0.1 % TFA (50 % v/v acetonitrile). After each extraction, resultant solutions were centrifuged at 10000 g for 5 min, supernatants were collected and combined followed by freeze drying. Each dried protein peptides were stored at 20 ºC until MALDI analysis.

Mass spectrometry analysis: MALDI-TOF analyses carried out as per reported method[7]. Briefly, an aliquot of 1 µL sample and 1 µL saturated HCCA matrix solution were mixed and spotted on MTP 384 steel target and dried. Mass spectra were obtained on ultraflex MALDI TOF/TOF equipped with pulse nitrogen laser (337 nm; 50 Hz) in reflectron positive mode.

Mascot protein identification: Tandem mass spectral data were used for database searching through Mascot program (Matrix Science Ltd., version 2.1) using 3.2 version of Bruker BioTools interface. Identification of proteins was made through Mascot Protein Search Database (MSDB) search engine (www.matrixscience.com) keeping *viridiplantae* (green plants) taxonomy. Trypsin was opted as proteolytic enzyme with carboxymethyl (C) as fixed modification and oxidation (M) as variable modification.

Bioactivity

Hydroxyl radical scavenging assay: This was performed using standard method[8]. Test was carried out in triplicates. BHT was used as positive control. The % inhibition was calculated by comparing optical density of test with positive control and negative control.

Scavenging activity of DPPH free radical: This activity was assayed to determine the antioxidant potential of the investigated plant protein as previously described[9]. Activity was calculated as follows: % of radical scavenging activity = 100 – [(each value - pos control)/neg control] × 100.

Lipid peroxidation inhibition assay: Inhibition of lipid peroxidation was performed as per the method of Kizil *et al.*[10]. RBCs were separated from anticoagulated blood and diluted to 1×10^6 cells/mL using phosphate buffer (50 mM, 120 mM KCl, pH 7.4). A 100 µL aliquot of these homogenous RBCs were mixed with plant protein of various concentrations (10-1000 µg/mL). BHT was used as standard.

Proteolytic activity: Protease activity assay was performed using standard method[11]. 2 % fat-free casein protein was used as substrate. To 0.5 mL of substrate, various concentrations (0-1000 µg) of *J. tanjorensis* crude protein sample were incubated for 2 h at 37 ºC. Assay was terminated by the addition of 0.44 M TCA and kept for incubation at room temperature for 0.5 h. Resulted solution was centrifuged to collect supernatant. To the supernatant collected, Lowry's reagent was added. Absorbance was read at 660 nm after 10 min.

Hemolytic activity: Direct hemolysis was performed as described by Boman and Kaletta[11]. HRBC (blood group A) was collected from a freshly collected anticoagulated blood sample. Various conc. (0-1000 µg/mL) of *J. tanjorensis* protein samples were added to 1 % HRBC in 1x PBS for 0.5 h at 37 ºC. The amount of hemoglobin released was measured spectrophotometrically at 540 nm.

Antimicrobial activity: Pure cultures of *Staphylococcus aureus* MTCC 96, *Staphylococcus aureus* MTCC 3160, *Pseudomonas aeruginosa* MTCC 741, *Pseudomonas*

aeruginosa MTCC 1688, *Bacillus subtilis* MTCC 441, *Escherichia coli* MTCC 723 were obtained from Microbial type culture collection, MTCC, Chandigarh, India. Nutrient agar, Nutrient broth, Muller Hinton Agar, Muller Hinton broth were obtained from Hi Media Private Ltd., Mumbai, India.

The pure cultures obtained were revived and maintained in nutrient agar at 37 ºC. The microorganisms were cultured in nutrient broth at 37 ºC overnight; the resulting suspensions were fixed to 0.5 McFarland standards, this is approximately equivalent to 1.5×10^8 CFU/mL.

Agar well diffusion method: Antibacterial activities of the proteins were determined by the formation of inhibition zone in Muller Hinton agar and compared with standard antibiotics. Briefly, Muller Hinton agar plates (25 mL) were prepared and the appropriate bacterial cultures were inoculated onto the surface of agar through sterile cotton swabs by spread plate method. Wells of 6 mm diameter were bored on the inoculated plates using sterile cork borer. 100 µL of samples were loaded to the wells, with concentration ranging from 250, 500, 750 and 1000 µg/100 µL. Ciprofloxacin hydrochloride (0.4 µg/100 µL) was used as standard antibiotic and 1x PBS (pH 7.4) was used as control. For each sample duplicates were done. Plates were incubated at 37 ºC for 24 h. Resulting zones of inhibition formed around the wells were measured.

Minimum inhibitory concentration (MIC): MIC of the protein samples were evaluated by broth micro-dilution method[13]. Briefly, two fold dilutions of the samples were prepared with Muller Hinton broth, that yields concentration ranging from 0.009, 0.019, 0.039, 0.07, 0.15, 0.3, 0.6, 1.25, 2.5, 5 mg/mL in a 96 well plate. Inoculated and non-inoculated well devoid of protein sample were used as positive and negative control, respectively. The least concentration detected with no visible bacterial growth has been considered as the MIC of the sample.

Cytotoxicity assay: Cytotoxicity was performed as per the method of Mosmann[13]. *Ehrlich ascites carcinoma* cells were suspended in RPMI 1640 complete medium (1×10^6 cells/mL) and each protein samples (0.005-2.5 mg/mL) were added and incubated for 24 h. MTT reagent solution was then added and incubated again at 37 ºC for 3 h. The coloured crystals generated were dissolved using DMSO and then read out at 530 nm. Percentage inhibition was then calculated.

Statistical analysis: For each assay, all values were normalized to control values and IC_{50} were calculated using Prism 5.1 Program at non-linear regression.

RESULTS AND DISCUSSION

Proteins belonging to different classes were identified by searching the mass spectrometry data through Mascot online protein search database. As these classes of proteins were known to be involved in antioxidant, antimicrobial and anticancer activities, the extracted proteins were evaluated for their biological activities with a view to develop it as a therapeutic agent.

2D-PAGE is the common method of choice for proteomic analysis followed by spot excision and MS analysis. This study presents substantial possibility in scientifically evaluating the indigenous plant resources for the isolation of therapeutically potential proteins. *J. tanjorensis* proteins which include DNA Binding proteins and other nuclear proteins identified using MALDI-TOF may provide a remarkable opportunity to develop drugs that would be effective against vast number of pathogenic microbes and effective against cancerous cells that in turn improves the quality of life.

2DE revealed more than 30 intense spots out of 89 identified spots (Fig. 1). These major spots were excised and were used for pre-processing followed by MALDI-TOF analysis.

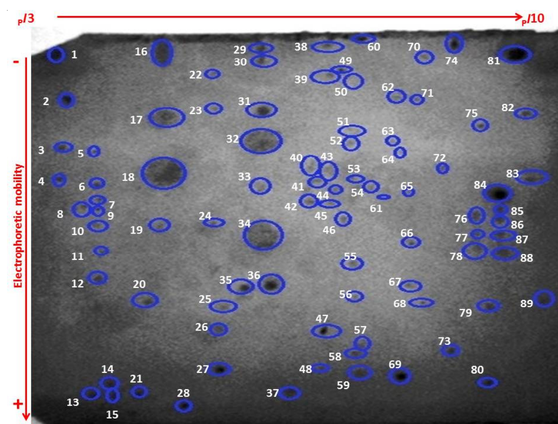

Fig. 1. 2DE pattern of *J. tanjorensis* proteins. Proteins spots are encircled and marked

MASCOT peptide mass fingerprinting search tool was used to identify various proteins using SwissProt database. 17 major proteins were identified. Out of these, spots 1, 18, 49 and 84 showed significant matches to major cancer related proteins DNA topoisomease, zinc finger CCCH domain, ATP dependent RNA helicase and actin-1 (Table-1).

Protein related to redox reactions were also identified as an abundant protein in the leaves of *J. tanjorensis*. In plants, the oxidase families carry multiple functions, such as oxidation of toxic reductants, biosynthesis and degradation of lignin in cell walls, defensive responses to wounding and against pathogen or insect and in auxin catabolism[14]. Another valuable proteins identified in the leaves of *J. tanjorensis* were Nuclear protein Ran-binding protein 1 (spot 69), DNA directed RNA polymerase (spot 36), RNA pseudourine repeat protein (spot 32) and RNA methyl transferase (spot 81).

Other proteins identified in *J. tanjorensis* belongs to Ribulose 1,5-*bis*phosphate carboxylase (spot 40) and peroxisomal (S)-2-hydroxy-acid oxidase (spot 34) which help in photorespiration and play its catalytic role in the oxidation of glycolate to glyoxylate and in the conversion of glyoxylate to oxalate through oxidation[15].

In addition, spot 16 corresponding to pleutropic drug resistance protein is known for its antifungal action and is abundant during slat stress. Major identified proteins in the present study are given in Table-1. Mascot score for most of the proteins were greater than 30 which indicates approximate homology with a significance level of $p > 0.05$. Although, medicinal property of *J. tanjorensis* has been reported in previous studies which were focusing mainly on the secondary metabolites extracted from this plant. Presently there is no scientific report on the primary metabolites of this plant (*J. tanjorensis*), which may contribute towards better medicinal and industrial usage of *Jatropha tanjorensis.* Proteins identified in the present study could be clustered into 8 functional groups (Fig. 2).

TABLE-1
DESCRIPTION OF VARIOUS PROTEINS IDENTIFIED USING MASCOT SEARCH

Spot No.	Biological process	Identified protein	Mascot score	Theoretical Mr
69	Stress response	Ran-binding protein 1	91	25814
36	Stress response	DNA Directed RNA polymerase	57	38985
34	Stress response	Peroxisomal Oxidase	56	40621
84	Stress response	Actin -1	58	42014
33	Stress response	Probable protein arginine N-methyltransferase	81	43823
18	Stress response	Zinc finger CCCH domain	58	50026
43	Stress response	Aspartic proteinase like protein 2	55	52536
40	Energy metabolism	RuBiSCO	55	52875
32	Energy metabolism	RNA pseudourine synthase	80	53702
51	Stress response	Oxidase	58	56369
17	Cytoskeleton constructing	Pentatricopeptide repeat protein	69	57570
31	Energy metabolism	Maturase K	69	59771
2	Cytoskeleton constructing	IAA synthase	55	68122
49	Stress response	ATP dependent RNA Helicase	59	81908
1	Stress response	DNA topoisomerase	68	103248
81	Hydrogen peroxide	RNA methyl transferase	42	105643
16	Salt stress/antifungal	Pleutropic drug resistance protein	42	163887

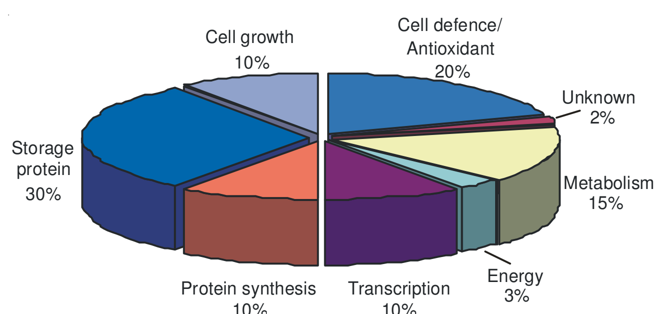

Fig. 2. Distribution of protein identified according to their major function in plant

Identification followed by bio-activity studies on plant proteins against various pathogenic microorganisms has attracted many researchers. Activity like antiproliferation is related with treating cancer. Scavenging of free radicals during oxidative stress has been implicated in treating human diseases like degenerative disorders and microbial pathogenesis. Present work has enlightened the vital role of *Jatropha tanjorensis* as an ethnomedicine. LPO inhibition (Fig. 3), OH^- reducing (Fig. 4) and DPPH scavenging (Fig. 5) ability has been identified as playing major roles in protecting cells against damage due to free radicals, radiation, carcinogens and xeno-biotics. Present work revealed that protein extract of *Jatropha tanjorensis* leaves possess significant anti-oxidant, antibacterial (against both gram stains bacterial species of different strains) activities

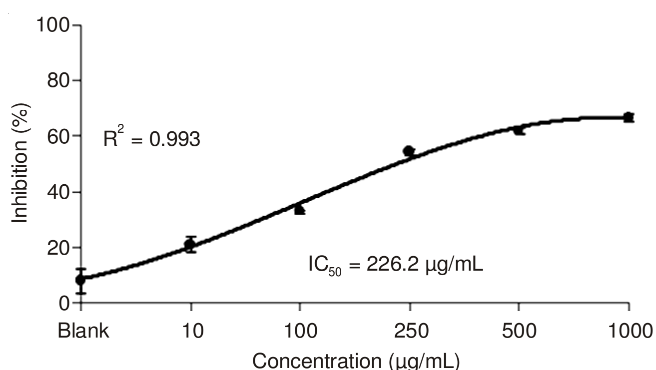

Fig. 3. LPO inhibition activity of *J. tanjorensis*

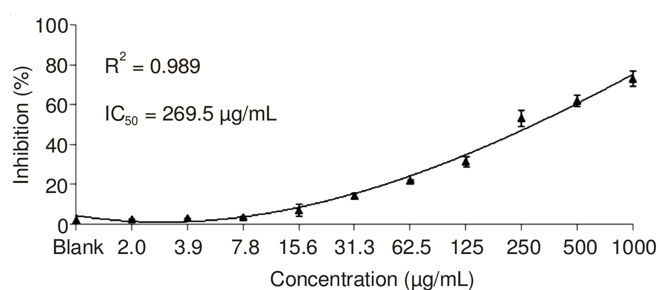

Fig. 4. Hydroxyl free radical inhibition activity of *J. tanjorensis*

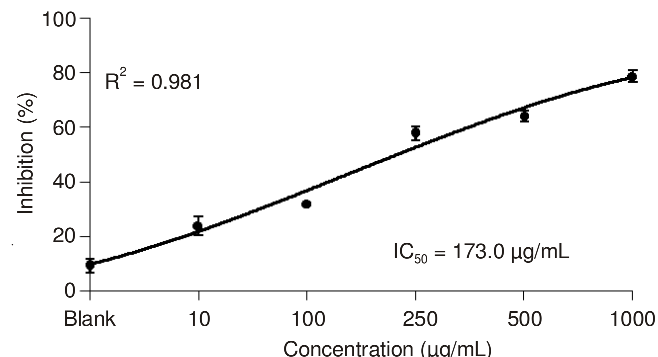

Fig. 5. DPPH free radical scavenging activity of *J. tanjorensis*

(Table-2, Fig. 6). Proteolytic (Fig. 7) and hemolytic (Fig. 8) activity were observed at higher concentration which suggest its non-toxic effect on normal physiological blood parameters. *J. tanjorensis* leaves protein extract has shown a potent anti-cancer potential against *Ehrlich ascites carcinoma* cells (Fig. 9).

Conclusion

The proteomic approach is attempted in the present work for *J. tanjorensis* to investigate the functional role of proteins and their therapeutic applications. Data obtained from the experimental work on *in vitro* studies such as antioxidant, antimicrobial and anticancer potentials provided substantial scientific evidences to support its ethnomedicinal claims against various patho-physiological conditions. This is the first report, wherein the advanced proteomic approaches have been explored to substantiate traditional claims of the medicinally important

TABLE-2
ANTIBACTERIAL ACTIVITY OF *J. tanjorensis* TOTAL PROTEIN EXTRACT

Microorganisms	Zone of inhibition (mm)		Average MIC (µg/100 µL)
	Total	CIP	ppt
S. aureus MTCC 96	25.12 ± 3.25	21.87 ± 2.5	7.8 ± 0.4
S. aureus MTCC EI	22.5 ± 1.58	18.5 ± 1.1	7.8 ± 0.3
P. aeruginosa MTCC 741	22.87 ± 3.7	15.5 ± 1.3	7.8 ± 1.0
P. Aeruginosa MTCC EI	27.75 ± 3	20.25 ± 0.5	7.8 ± 1.1
B. subtilis MTCC 441	19.62 ± 2.3	23.75 ± 0.9	7.8 ± 0.7
E. coli MTCC 723	27.25 ± 3.23	25.25 ± 2.3	7.8 ± 0.2

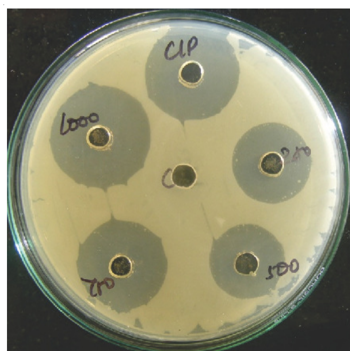

Fig. 6. Representative image of agar well diffusion assay. *S. aureus* MTCC 96 susceptibility towards *J. tanjorensis*

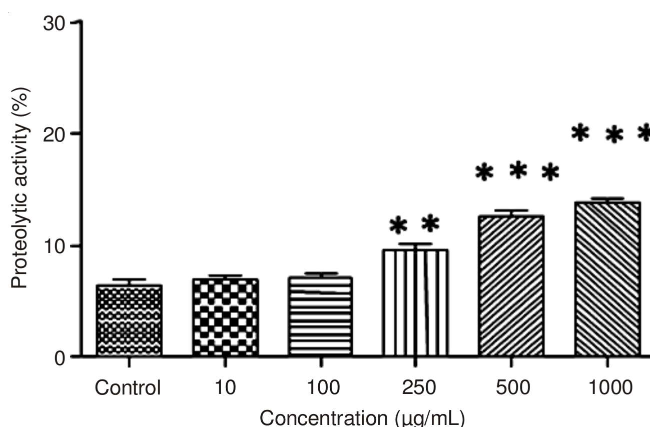

Fig. 7. Proteolytic activity of *J. tanjorensis* leaves total protein

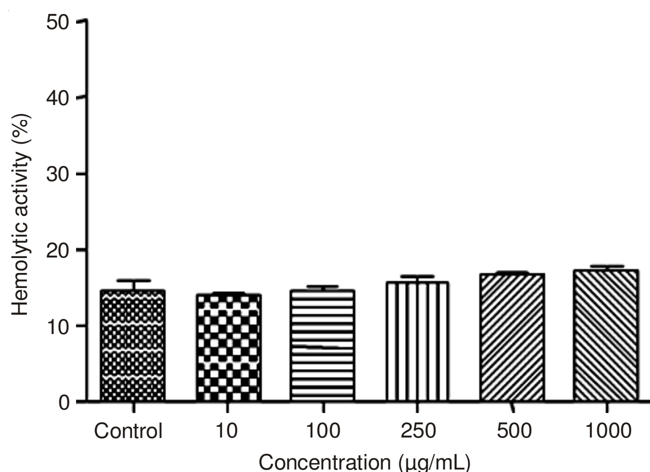

Fig. 8. Hemolytic activity of *J. tanjorensis* leaves total protein

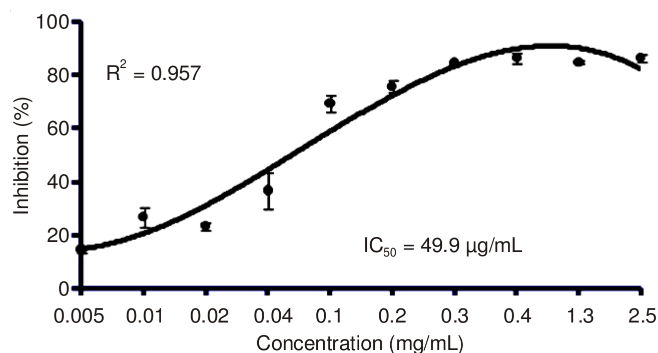

Fig. 9. Anticancer activity of *J. tanjorensis* leaves total protein against EAC

plants and subsequently contribute towards the development of drugs for the better healthcare of human society.

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