Asian Journal of Chemistry; Vol. 27, No. 9 (2015), 3249-3253

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2015.18472

Investigations into Phenolic and Alkaloid Constituents of *Jatropha tanjorensis* by LC-MS/MS and Evaluating its Bioactive Property

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Received: 18 July 2014; *Accepted*: 21 April 2015; *Published online*: 26 May 2015; AJC-17234

Jatropha tanjorensis (Euphorbeacea) leaves are used as a food and for various herbal medicines in India and African countries. But only diminutive information is available on the secondary metabolites and their correlation with its traditional uses. Reversed-phase Liquid chromatography with C18 bonded silica gel coupled to ultraviolet (UV) detection and mass spectrometer encompass of quadrupole mass analyzer with time-of-flight mass detection (micrOTOF-QII) was standardized for the identification of semipolar compounds in the extract. Total ion chromatogram (TIC) was processed using Hystar 3.2 software tools. Compounds were assigned by comparing their mass signals with compounds already reported and MS/MS spectral MassBank libraries. With this approach several compounds not reported previously from *Jatropha tanjorensis* were identified. The methanolic extract from leaves of *Jatropha tanjorensis* (MEJT) contained a complex mixture of 20 flavonoids predominantly as glycosides, with few aglycones. Isorhamnetin-3-glucoside-4'-glucoside, delphinidin-3-O-2''-O-β-xylopyranosyl-β-glucopyranoside, 6-c-hexosyl-8-c-pentosyl apigenin, cyanidin-3,5-di-O-glucoside, 3',7-dimethoxy-3-hydroxyflavone and 2'',3'',4',5,6'',7-hexa-O-methylisovitexin were found in the methanolic extract of *J. tanjorensis* leaves. The core aglycones of these flavonoids were identified as apigenin, luteolin, isorhamnetin, quercetin and kaempferol. Few other bioactive molecules like rhein, benzamidine and derivative of ellagic acid were also identified. Apart from phenolic compounds few medicinally potent alkaloids like norharman, harmane, salsolinol and anabasine were also identified. To depict its biological activity against cancer cells, inhibition of proliferation assay (MTT) was performed on human skin carcinoma cells (A431) with an IC_{50} of 58.53 µg/mL. The methanolic extract from leaves of *Jatropha tanjorensis* has shown potent antimicrobial activity against various strains of bacteria with an MIC of 7.8 µg/mL. The methanolic extract from leaves of *Jatropha tanjorensis* was also evaluated for its anti-inflammatory activity through proteinase inhibition activity (IC_{50} 166.2 µg/mL), inhibition of protein denaturation (IC_{50} 165.4 µg/mL) and membrane stabilization (IC_{50} 160.4 µg/mL). Antioxidant property was evaluated using reducing hydroxyl ion assay with an IC_{50} of 46.43 µg/mL. Results acquired prove the potent efficiency of selected plant against various disease conditions progresses to cancer. Present study also provide the necessary information on the chemical composition of *Jatropha tanjorensis*, thus suggesting yet another "authentication" in the identification of the selected plant.

Keywords: *Jatropha tanjorensis*, LC-MS/MS, Flavonoids, DPPH, MIC, *in vitro*, MTT.

INTRODUCTION

Jatropha tanjorensis (Family, Euphorbiaceae) also known as the "Catholic vegetable" or "Reverend Father's vegetable" is an exotic plant species found in India, Africa and America. Ethnomedicinally *tanjorensis* was used as a vegetable to make palatable soup, antiseptic and antihypertensive and there have also been reports on this plant being used as a remedy against diabetes[1]. Nutraceutical studies were performed earlier by our group which proves the potent role of this herbal drug as a source of nutritional medicine[2].

The characterization of plant metabolites is an important concern not only in phyto-chemistry but also in phyto-physiology. A pharmacognostic & phytochemical analysis typically needed to generate database for herbal drug authentication and its quality assessment. Also, this help in defining suitable marker compounds and predicting their related biological activity. Among the main constituents in plants, aglycone flavonoids, flavone glycosides and alkaloids have attracted a special attention. Flavonoids have numerous physiological activities which are well known and have been extensively studied. Clinical experiments on flavonoids have shown its potent biological activity against various diseases[3]. Flavonoids are well known for its natural non-toxic antioxidant property, possessing free radical scavenging and chelating properties, this makes flavonoids as potential bioactive molecules in combating diseases caused by free radicals such as ischemia, anemia, arthritis, asbestosis and its related

secondary diseases like cancer. Earlier studies have shown the potent role of *J. tanjorensis* as an antioxidant[4].

Qualitative identification of various phytoconstituents in *J. tanjorensis* methanolic extract, mass spectrum analysis was performed using ESI-LC–MS/MS with Q-II analyzer and TOF (time-of-flight) detector. Electrospray ionization mass spectrometry (ESI-MS) is a soft ionization technique, providing necessary molecule structure related information. MS/MS fragmented pattern helps in distinguishing between closely related flavonoids, this technique also provide suggestions on the position of glycosylation. Searchable MS/MS spectra libraries *e.g.*, Mass Bank, Metlin-Scripps, MS/MS fragment viewer were used to interpret the results of liquid chromatography interfaced with electrospray ionization (ESI-Q-II TOF) mass spectrometer. Hence, the purpose of the present research was to identify the various bioactive phytoconstituents in their native state form in *Jatropha tanjorensis* through LC-MS/MS fragmentation pattern and to evaluate its anticancer property using A431 cancer cells. Antibacterial, anti-inflammatory and antioxidant potential were also carried out to prove its versatility activity against various diseases.

EXPERIMENTAL

Analytical reagent grade, HPLC-grade chemicals and MilliQ water (Millipore, Bedford, MA) were used for each assay.

Pure cultures of the bacteria *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.

ESI calibrant: ESI calibrant was purchased and used as an internal calibrant (Sigma-Aldrich Chemie GmbH Co., St. Louis, MO). Analyses of the internal calibrant were done as per the manufacturer instruction upto 0.60 ppm correction at both polarities.

Jatropha tanjorensis (Euphorbiaceae) leaves were collected in and around SASTRA University, Thanjavur, India during January 2012. For the authentication purpose, herbarium voucher specimen was prepared and identified with the deposited specimen in Raphinet Herbarium (1921) St. Joseph's College, Trichy, India. Pharmacognostic features were studied extensively and respective parameters were used for its standardization and authentication of the plant drug used[2].

Extraction procedure: Fresh leaves of *J. tanjorensis* were dried and powdered (100 g), which was then defatted using petroleum ether, after which the powder was extracted successively in increasing polarity solvents, finally the left out residue was extracted with methanol at room temperature for 72 h. The resultant extract was dried in a vacuum rotavapor at 40 °C. This crude methanolic extract from leaves of *J. tanjorensis* (MEJT) was used directly for flavonoids identification using LC-MS/MS. For alkaloid identification, a part of this crude methanolic extract was filtered and filtrate was collected and evaporated, this fraction was then mixed with 100 mL of 1 M HCl until homogenous solubility. This was then employed for liquid-liquid extraction step using 100 mL of chloroform. Aqueous phase was alkalized using dilute 25 % ammonia and further extracted with 50 mL chloroform for three times. Organic layer was taken and concentrated, resulted in 1.2 g of a dark brown crude alkaloid extract. This solution was analyzed using LC-ESI-MS for the identification of the alkaloids.

Sample preparation: Crude methanolic extract sample was weighed and dissolved in methanol. Crude alkaloid fraction was also taken separately and re-dissolved in acetonitrile to get 1 mg/mL concentration. 500 µL of each solution was taken for LC-MS analysis.

LC-MS/MS: Phytoconstituents were separated on a reverse-phase Acclaim 120, RP-C18 120 Å, 2.1 × 150 mm, 3.0 µm column (Dionex, USA), held at 30 °C. Molecules were eluted using acetonitrile (A) and 1 % aqueous formic acid v/v (B) as mobile phase with a discontinuous gradient; starting with 95 % B for 10 min, to 90 % B after 1 min, to 60 % B in the next 9 min, next 10 min B reaches 80 %, next 10 min to reach 40 % B, 5 min to reach 0 % B and was maintained for another 10 min until the run ends, with flow rate at 0.2 mL/min. Chromatographic profiles were acquired at 356 nm wavelength. Injection volume was 50 µL. Eluted components were ionized by electrospray ion source (ESI) in negative mode for MJET and positive mode for crude alkaloid fraction, using N_2 for nebulization (30.2 psi) and drying (flow of 6 L/min, temperature of 280 °C). Set capillary voltage was 4500 V, end plate offset was set at -500 V, energy transfer time of 80.0 µs, collision cell RF 350.0 Vpp, pre pulse storage of 5.0 µs. AutoMSn mode was chosen to detect and identify the best MS/MS peaks for analysis using DataAnalysis software provided by the manufacturer. Scan mode in *m/z* range of 50-1500 *m/z*.

Bioactivity

Inhibition of protein denaturation: Reaction mixture (0.5 mL) contain 250 µL 5 % aqueous bovine serum albumin and 50 µL of different concentrations (50, 100, 250, 500 and 1000 µg/mL) of methanolic extract from leaves of *Jatropha tanjorensis*, 1N HCl was used to adjust pH at 6.3. Samples were then mixed gently and incubated at 37 °C for 15 min followed by heating for 3 min at 57 °C. This mixture was then brought to room temperature followed by addition of 650 µL PBS (1x, pH 6.3). Turbidity formed was measured spectrophotometrically at λ = 660 nm, 50 µL milli-q water was used in place of extracts for control test. Product control was devoid of protein. The percentage of protein denaturation inhibition was estimated using:

$$\text{Inhibition (\%)} = \left(100 - \frac{\text{O.D. of test} - \text{O.D. of product control}}{\text{O.D. of control}}\right) \times 100$$

The control normalized to 100 % protein denaturation. Acetyl salicylic acid at 250 µg/mL was used as positive control.

Effect on membrane stabilization: 1 mL of reaction mixture comprise of 300 µL 0.25 % NaCl solution (hypotonic saline), 100 µL of pH 7.4 phosphate buffer (0.15 M), 100 µL of extract solution at 50, 100, 250, 500 and 1000 µg/mL concentration in 0.9 % saline and 500 µL of 10 % human RBC. 100 µL of 0.25 % NaCl was used in place of test solution as a

control test, while product control devoid of RBCs. Each tubes were then incubated at 56 °C for 0.5 h followed by cooling the tubes in water bath for 20 min. All tubes were then centrifuged for 10 min at 1500 rpm. Supernatant was collected from each tube kept in freshly labeled tubes separately and absorbance was read at λ = 560 nm. Membrane stabilizing activity was calculated as earlier.

Proteinase inhibitory activity: 2 mL of reaction mixture comprised of trypsin (0.06 mg), 100 µL of Tris-HCl buffer (25 mM; pH 7.4) and 100 µL (50, 100, 250, 500 and 1000 µg/mL) of MEJT. Each tube were then mixed gently and incubated at 37 °C for 5 min followed by addition of 1 mL casein (0.8 % w/v) followed by incubation for an additional 15 min followed by stopping reaction with the addition of 800 µL 70 % (v/v) perchloric acid. Tubes were centrifuged and supernatant was collected and absorbance was read at 280 nm against buffer as blank. The percentage of inhibition was calculated as earlier.

Hydroxyl radical scavenging assay: Hydroxyl radical scavenging assay was performed as described somewhere[5].

Antibacterial activity: The pure cultures obtained were revived and maintained in nutrient agar at 37 °C. The microorganisms were cultured in nutrient broth at 37 °C overnight; 0.5 McFarland standards was used which is approximately equivalent to 1.5×10^8 CFU/mL.

Agar well diffusion method: The antimicrobial potential of the methanolic extract from leaves of *Jatropha tanjorensis* was estimated by the formation of inhibition zone in Muller Hinton agar compared to antibiotics. Briefly, Muller Hinton agar plates (25 mL) were prepared and the appropriate bacterial cultures were inoculated over the surface of the agar using 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 in triplicates. Ciprofloxacin hydrochloride (0.4 µg/100 µL) was used as standard antibiotic and PBS (pH 7.4) was used as control. The plates were then incubated for 24 h at 37 °C and the resulting zones of inhibition across wells were noted.

Minimum inhibitory concentration (MIC): Minimum inhibitory concentration of methanolic extract from leaves of *Jatropha tanjorensis* was evaluated using broth microdilution method[6]. 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. Uninoculated and inoculated well devoid of protein sample were used as negative and positive control respectively. The least concentration detected with no visible bacterial growth has been considered as the MIC of the sample.

Anticancer assay: A431 cells were treated with various concentrations of MEJT for 48h under 37 ºC and 5 % CO_2 in CO_2 incubator. MTT assay was performed in accordance with standard textual method[7]. Absorbance was read out at 590 nm using Epoch microplate spectrophotometer (BioTek, USA).

RESULTS AND DISCUSSION

In this study, a method was successfully developed using a stationary phase with hydrophilic endcapping (C18) coupled with MS (quadrupole II-TOF) for the determination of various compounds present in methanolic extract from leaves of *Jatropha tanjorensis*. As can been seen from Figs. 1 and 2, baseline separation was achieved for twenty major compounds. LC-MS chromatogram of methanolic extract from leaves of *Jatropha tanjorensis* showed various levels of peaks which were further analyzed to identify the eluted analytes based on their exact mass. Table-1 represents the retention time, *m/z* ratio and respective fragmented ion masses of molecule identified in methanolic extract from leaves of *Jatropha tanjorensis*. Alkaloids were identified at positive ionization mode with their base peaks assigned as $[M+H]^+$, similarly at negative ionization mode *m/z* were assigned as $[M-H]^-$ which helps in the detection of flavonoids and other phenolics molecules.

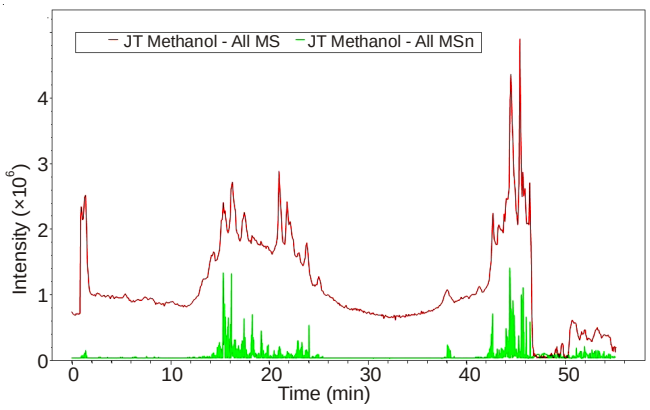

Fig. 1. Total ion chromatogram (TIC) with MS and MSn spectrum of methanolic extract of *Jatropha tanjorensis*, as monitored during both polarity

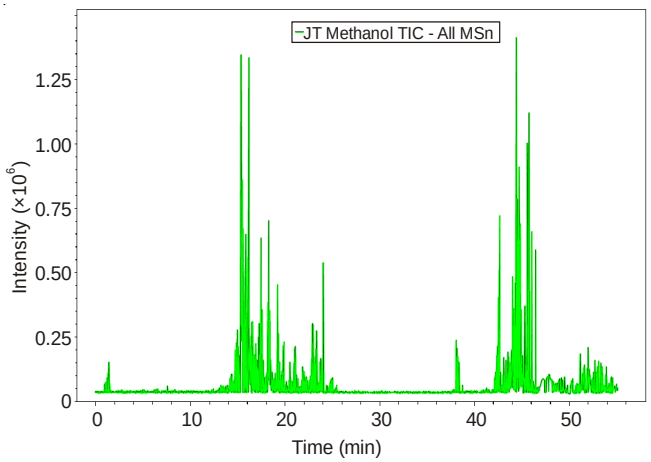

Fig. 2. Total ion chromatogram MS/MS of methanolic extract of *Jatropha tanjorensis*

Phytochemical investigation on methanolic extract of *Jatropha tanjorensis* leaves led to the identification of flavone glycosides like isorhamnetin-3-glucoside-4'-glucoside, delphinidin-3-O-2″-O-β-xylopyranosyl-β-glucopyranoside, 6-c-hexosyl-8-c-pentosyl apigenin, 3',7-dimethoxy-3-hydroxy-flavone and 2″,3″,4',5,6″,7-hexa-O-methylisovitexin with few aglycone flavonoids as apigenin, luteolin, isorhamnetin, kaempferol and quercetin were also identified in negative mode. Molecules like rhein a potent antibacterial phytomolecule, benzamidine known protease inhibitor, derivatives of cyanidin and ellagic acid is potent antioxidant were also identified. Apart from phenolics compounds few medicinally potent alkaloids

TABLE-1
ESI-MS AND ESI-MS/MS PRODUCT IONS OF FRACTIONS FROM METHANOLIC EXTRACT OF *Jatropha tanjorensis* LEAVES

S. No.	Identified compound	Retention time (min)	Mass	MS (parent ion)	MS/MS (product ion)
1	Isorhamnetin-3-glucoside-4'-glucoside	24.9-25.0	640	639	285, 300, 315, 225, 339, 369, 431, 624
2	Delphinidin-3-O-(2"-O-β-xylopyranosyl-β-glucopyranoside)	15.5-15.6	597	596	271, 313, 297
3	2",3",4',5,6",7-Hexa-O-methylisovitexin	18.0	516	515	311, 283, 269, 394, 323, 123, 351
4	6-c-Hexosyl-8-c-pentosyl apigenin	14.8	580	579	369, 399, 339, 429, 381, 411, 312, 441, 459, 205, 353
5	Kaempferol	27.8-27.9	286	285	199, 211, 201, 215, 227, 239, 256, 269
6	3',7-Dimethoxy-3-hydroxyflavone	44.1-44.5	298	297	183, 119, 199
7	Isorhamnetin	21.3-21.5	316	315	271, 243, 227, 203, 300, 255, 215
8	Cyanidin-3,5-di-O-glucoside	18.4-18.8	610	609	327, 313, 357, 298, 207, 192, 285, 447
9	Apigenin	20.6-20.8	270	269	117, 151, 169
10	Luteolin	19.4-19.6	286	285	133
11	Quercetin	19.7-19.8	302	301	151, 179, 183, 245
12	Rhein	43.4-43.5	284	283	183, 269, 119
13	Benzamidine	11.8	120	121	77
14	Ellagic acid derivative	20.7	658	657	257, 327, 214, 301, 242
15	Norharman	11.0-11.2	168	169	115, 168, 140, 128
16	Harmane	19.7-19.8	182	183	115, 140
17	Salsolinol	11.6-11.7	179	180	115, 140
18	Anabasine	9.9	162	163	144, 129, 107, 112, 118, 115

like norharman, harmane, salsolinol and anabasine were also identified in positive ionization mode.

Total ion chromatogram (TIC) profile including MS/MS chromatogram is shown in Fig. 2, which shows that most of the phytomolecules were eluted out from 10-20 min and then later at 45-50 min. Various derivative forms of flavonoids were identified by matching *m/z* values and MS/MS pattern to those molecules available in various online available databases. The presence of double the mass of vitexin, apigenin glycoside, luteolin glycoside with exact MS/MS fragment pattern suggested us the presence of dimeric forms of these flavonoids[8-10].

Extracts rich in flavonoids and alkaloids are known to provide numerous therapeutic activities which are in concurrence with the folklore usage of *J. tanjorensis* as an edible and antiseptic medicinal plant.

The result from zone of inhibition and MIC assays reveals that methanolic extract from leaves of *Jatropha tanjorensis* is effective against both gram strains bacteria's all the bacterium selected in this study. Methanolic extract from leaves of *Jatropha tanjorensis* was most effective against *Escherichia coli* MTCC 723 as depicted from zone of inhibition assay with a zone of inhibition diameter of 25.25 ± 1.66 mm. MIC assay reveal that 7.8 µg/mL is sufficient enough to inhibit bacterial culture which is comparable to MIC values of standard antibiotics used (Table-2 and Fig. 3).

TABLE-2
ANTIMICROBIAL ACTIVITY OF MEJT
AGAINST VARIOUS BACTERIAL STRAINS

Microorganisms	Diameter (mm)
Staphylococcus aueus MTCC 96	17.37 ± 1.88
Staphylococcus aureus MTCC 3160	18.75 ± 2.17
Pseudomonas aeruginosa MTCC 741	16.25 ± 3.23
Pseudomonas aeruginosa MTCC 1688	21.37 ± 2.06
Bacillus subtilis MTCC 441	23.25 ± 1.66
Escherichia coli MTCC 723	25.25 ± 1.66

n = 3 ± SD

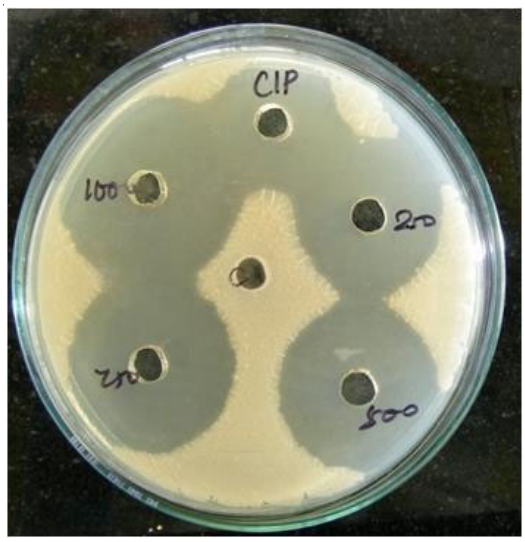

Fig. 3. Representative image of antimicrobial activity of methanolic extract from leaves of *Jatropha tanjorensis*

It is now well known fact that denaturation of the protein is one among the basis of autoimmune disorder such as rheumatoid arthritis. In the present study stabilization of RBC membrane at different conditions were also carried out, this may be correlated to lysosomal membrane stabilization. In a similar way neutral serine proteinases of lysosomal granules present inside the neutrophils are considered as rich sources of proteinases and are one of the causative agents for arthritic reactions. It was clearly observed that proteinase inhibition, protein denaturation inhibition and RBC membrane stabilization from selected plant extract has shown anti-inflammatory activity which can be associated against damaging of tissue due to external factors. Results are compiled in Table-3.

Flavonoids are well known for its involvement against chronic diseases due to its antioxidant potentials[11,12]. Antimicrobial activity[13] and anti-inflammatory activities[14] of flavonoids and

TABLE-3
ANTIINFLAMMATORY ACTIVITY OF *Jatropha tanjorensis* (MEJT). ACETYLATED SALISIALIC ACID WAS USED AS A POSITIVE CONTROL

Treatment (µg/mL)		Inhibition of protein denaturation (%)	Membrane stabilization (%)	Proteinase inhibition (%)
Crude methanol extract	50	25.0 ± 1.1	24.1 ± 0.8	27.3 ± 1.2
	100	47.5 ± 1.0	48.4 ± 0.7	49.9 ± 1.3
	250	73.4 ± 1.1	76.2 ± 1.1	77.9 ± 0.8
	500	83.8 ± 2.1	85.8 ± 1.7	89.6 ± 1.5
	1000	92.6 ± 1.4	95.1 ± 2.1	97.2 ± 1.0
IC_{50} (µg/mL		165.4	160.4	166.2

n = 3 ± SD

alkaloids are also well established. Scavenging of hydroxyl ion free radical is a direct method to investigate the antioxidant potential of any compound or plant extracts[15]. Fig. 4 represents the quantity of methanolic extract from leaves of *Jatropha tanjorensis* required to provide 50 % inhibition (IC_{50}). The crude methanolic of selected plant exhibited a noticeable OH^- reducing capacity with an IC_{50} of 46.43 µg/mL. Methanolic extract also revealed the potent cytotoxic activity against A431 cancerous cells with an IC_{50} value 58.53 µg/mL.

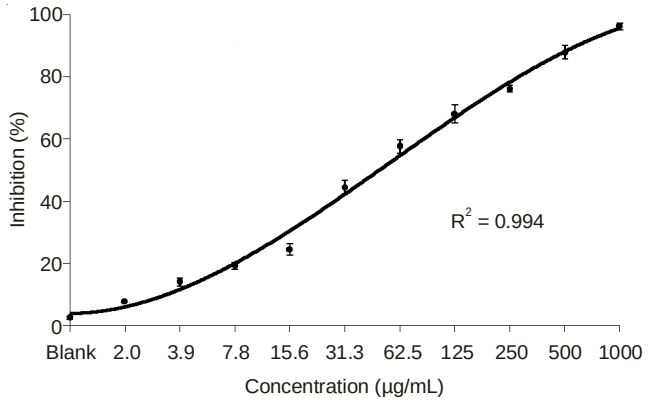

Fig. 4. Reducing capacity of methanolic extract from leaves of *Jatropha tanjorensis* showing an IC_{50} of 46.43 µg/mL; n = 3 ± SD

Conclusion

The presence of apigenin and luteolin derivatives were never stated from *J. tanjorensis* and this provides an understanding on the possible role of these flavonoids and its derivatives in the biological activity connected with the traditional use of *Jatropha tanjorensis* leaves. Similarly few known alkaloids were also identified and reported for the first time. Fragment pattern of these molecules specially with essential A and B and C-ring fragments are characteristic for every different type of flavonoids hence provide a typical identification pattern for each class of these molecules. The qualitative technique illustrated in the presented research paper is also easy and provide a better way to identify molecules from crude extract with less pre-processing steps. The present study represents the first inclusive report on the phytoconstituents of traditional plant *Jatropha tanjorensis* and therefore enlightens the traditional uses of *J. tanjorensis*. Present work also provides a more reliable authentication in the identification of the selected plant.

ACKNOWLEDGEMENTS

The authors thank Vice-Chancellor, SASTRA University for his constant encouragement. Financial support from the DST (VI-D&P/267/08-09/TDT), Government of India for the purchase of LC-MS/MS instrument is gratefully acknowledged.

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