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Research Article

A PRELIMINARY STUDY ON THE ANTIPROLIFERATIVE ACTIVITY OF *NYCTANTHES ARBORTRISTIS* LEAF EXTRACTS USING FISSION YEAST AS A MODEL

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A B S T R A C T

The aim of the present study was to evaluate the total phenolic content, antioxidant activity and antiproliferative activity of crude extracts of *Nyctanthes arbortristis*. The extracts were prepared by three experimental conditions (25 °C, elevated temperature and ultrasound waves) and four solvent systems [Aqueous, Methanol, Water: Methanol (1:1) and Chloroform: Methanol (1:1)]. For screening of antiproliferative activity, a fission yeast *Schizosaccharomyces pombe* was used as a model. MTT assay was used for estimating the cell viability and the results were expressed as % growth inhibition. The highest phenolic content was estimated in the methanol and chloroform: methanol extracts. The antioxidant capacities measured using DPPH scavenging activity and phosphomolybdenum assay showed that the methanol and chloroform: methanol extracts had strong antioxidant capacity. Based on the MTT assay performed on yeast cells, Water: Methanol, Chloroform: Methanol and Methanol extracts showed higher antiproliferative activity than the positive control. The results of the experiments show that decrease in the cell viability was in a dose dependent manner. In conclusion, this study has indicated that *Nyctanthes arbortristis* extracts possess strong antioxidant and antiproliferative activity. Furthermore, it could act as a potential source for identifying new molecules with chemotherapeutic potential against cancer.

INTRODUCTION

Medicinal plants have been used since ancient times for the treatment of various diseases. India is a rich source of medicinal plants which have contributed greatly to the treatment of disorders through its ancient traditional medical systems such as Ayurveda, Unani and Siddha (Tariq *et al*., 2017). The presence of different classes of phytochemicals or secondary metabolites is responsible for the medicinal properties of the plant. Phenolic compounds present in plants are known to possess antioxidant and cancer chemopreventive properties (Soobrattee & Neergheen, 2005; Stoner & Mukhtar, 1995).

As per the prediction of The International Agency for Research on Cancer GLOBOCAN project, India's cancer burden will nearly double in the next 20 years, from slightly over a million new cases in 2012 to more than 1·7 million by 2035 (Mallath *et al*., 2014). Among males, lung cancer is the leading cause of cancer mortality, accounting for 12% of all cancer deaths across the world (Ferlay *et al*., 2015).

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It is now well known that different patients respond in different ways to the same medication as genetics plays an important role in determining drug response. As a consequence, a single drug does not have the identical effectiveness in different patients (Evans and McLeod, 2003). For this reason, it has become necessary to widen the range of chemotherapeutic agents so that patients can be treated based on the genetic alterations. Plants have been a source of anticancer drugs and have provided us with many leads with potential to become new chemotherapeutic drugs. Some examples of clinically used anticancer drugs derived from medicinal plants are vincristine, vinblastine, paclitaxel and others (Cragg and Newman, 2005). Therefore, exploring the medicinal property of plants would help in identification of new molecules with anticancer activity.

Nyctanthes arbortris-tis Linn belonging to family Oleaceae has immense medicinal potential and different parts of this plant are used by Ayurveda, Unani and Siddha traditional medicinal systems for treatment of various disorders (Rani *et al*., 2012). It is known to possess several pharmacological activities like immunotoxic, antiallergic, antihistaminic, purgative, antibacterial and ulcerogenic (Agrawal and Pal, 2013). Additionally, preliminary reports are available which suggest the anticancer potential of this plant (Khatune *et al*.,

2003). Despite having anticancer potential, no bioactive compound has been isolated from this plant.

Thus, in the present study, we have investigated the antioxidant activity and antiproliferative activity of this plant. The antiproliferative activity was determined using fission yeast *Schizosaccharomyces pombe* as a model organism. *S. pombe* has a cell division pattern similar to mammalian cells (Lee *et al*., 2012). Moreover, basic mechanisms such as DNA metabolism and cell proliferation control are conserved between yeast and human cells (Sánchez-Picó *et al*., 2014). Hence, our study used fission yeast mutant having an altered cell cycle to perform a pilot screen on a range of extracts prepared from the plant. Because of its altered cell cycle, this mutant has rapid proliferation rate and hence it mimics a cancer cell.

MATERIALS AND METHODS

Chemicals

Methanol, Chloroform, Sodium carbonate and Dextrose were procured from Merck chemicals. Dimethyl Sulphoxide (DMSO), 3-(4, 5-dimethylthiazol-2yl) 2, 5-diphenyltetrazolium bromide (MTT) dye, Gallic acid, Folin-Ciocalteu reagent, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and Yeast extract were procured from HiMedia Laboratories Pvt. Ltd. Paclitaxel was procured from Hetero Healthcare. Peptone was obtained from Sisco Research Laboratories Pvt. Ltd. Ascorbic acid was ordered from S. D. Fine Chemicals. All chemicals used in the experiments were of analytical grade. The bath sonicator used in the study was from Chicago electric power tools, temperature controlled water bath was from Shivtronics and rotary shaker was from Bright Instruments.The Multiskan Go plate reader was purchased from Thermo Fischer Scientific.

Collection of plant material and extraction

The leaf part of *Nyctanthes arbortristis* was collected from Xavier Residence c/o St. Xavier's College Campus, Ahmedabad. The plant was verified by Dr. Hitesh Solanki from Botany Department of Gujarat University. Leaves were thoroughly washed with tap water to remove the dirt particles followed by distilled water and were then shade dried. The dried leaf was then crushed into fine powder and subjected to extraction. The crude extracts were prepared by using three experimental conditions: (i) elevated temperature, (ii) 25 ºC temperature and (iii) Sonication. The elevated temperature was carried out below the boiling point of respective solvent used for extraction. Sonication was performed using ultra sound waves with aid of bath sonicator. The solvent systems Water, Methanol, Water: Methanol (1:1) and Chloroform: Methanol (1:1) were utilized for extraction. The purpose of using different methods and solvents was to ensure effective extraction of phytochemicals from plant material. The powder to solvent (w/v) ratio was kept 1:10. For hot and sonication extraction, one gram powder was taken in stoppered tube and 10 ml of solvent was added to it which was then incubated in water bath or bath sonicator for one hour. At the end of the incubation period, the filtrate was collected in a pre-weighed beaker. The extraction was performed in this manner until the solvent became colourless. In case of cold extraction, the solvents were changed every 24 hour and constant agitation was provided at 100 rpm. The extracts were filtered using Whatman no. 1 filter paper. The collected filtrate was pooled and evaporated to dryness until a constant weight was achieved. Then stocks of the extract at concentration of 100 mg/ml were prepared in DMSO and stored at -20 ºC until further use.

Phytochemical analysis

Estimation of Total Phenolic content

Total phenolic content was estimated by the Folin-Ciocalteu method as described by Herald *et al*., (2012), with slight modifications. Briefly, 20 µl plant extract was added into the well and to this 80 µl Folin Ciocalteu reagent was added. At the end of the 6th minute, 100 µl of 7.5% Na_2CO_3 was added to the mixture. The plate was incubated in the dark for 90 minutes. The absorbance was measured at 765 nm in a MultiskanGo plate reader. Gallic acid was used as a standard to generate a calibration curve (12.5 µg/ml to 200 µg/ml). The results were expressed in terms of gallic acid equivalent (mg GAE/g extract).

Antioxidant assay

Free radical scavenging activity using 2, 2-diphenyl-1-picrylhydrazyl (DPPH)

The free radical scavenging activity of the extracts was estimated using free radical DPPH. A freshly prepared 0.1 mM DPPH in methanol was used for the estimation. 25 µl of plant extract (250 µg/ml) was added to a 96 well plate. To this 200 µl DPPH was added and incubated for 30 minutes. The absorbance was measured at 517 nm at the end of the incubation period using Multiskan go plate reader. A standard curve was prepared using Ascorbic acid (10µg/ml to 50 µg/ml). The results were expressed as percentage free radical scavenging activity. Appropriate blank and controls were also used in the experiment. The following formula was used to calculate % DPPH quenching:

$$\textbf{\% DPPH quenched} = [1 - (A_{sample} - A_{blank}) / (A_{control} - A_{blank})] \times 100 \qquad (1)$$

Total antioxidant activity by Phosphomolybdenum assay

The total antioxidant capacity was performed as per the method described by Prieto *et al*., 1999. This method is based on the reduction of Mo (VI) to Mo (V) and formation of a green phosphate/Mo (V) complex at acidic pH. The method followed was: 0.1 ml plant extracts (1 mg/ml) were taken in a tube and 1 ml phosphomolybdenum reagent (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) was added to this. The samples were then incubated at 95 ºC for 90 minutes in a water-bath. After the incubation period was over, samples were allowed to cool at room temperature. The absorbance of the samples was measured at 695 nm. A standard curve was prepared using Ascorbic acid (50 µg/ml to 250 µg/ml). The results were expressed as mg Ascorbic acid equivalents per gram of dry extract.

Yeast culture and MTT assay

Schizosaccharomyces pombe was procured from the National Collection of Yeast Cultures (NCYC 1683). Yeast cells were cultured in YPD medium (1% Yeast extract, 2% Peptone and 2% Dextrose) and incubated overnight at 25 ºC. The antiproliferative activity of the extracts was measured by 3-(4, 5-dimethylthiazol-2yl) 2, 5-diphenyltetrazolium bromide (MTT) assay. Briefly, the absorbance of an overnight incubated culture was read at 600 nm to measure cell growth.

The culture was diluted to achieve desired cell concentration and the cells were then seeded to a 96 well plate. The cells were treated with different amount of the extracts (250, 500 and 1000 µg) from stocks and incubated for 24 hours. Paclitaxel was used as a positive control in the experiment (Schiller *et al*., 2002). DMSO was used as a vehicle control and untreated cells were taken as negative control. Following the incubation period, MTT dye (5 mg/ml) was added to the wells and incubated for four hours in the dark. The formazan crystals formed at the end of the incubation period were dissolved in DMSO. The absorbance was read at 570 nm using Multiskan Go microplate reader (Thermo Fisher). The percentage cell viability was calculated using the following formula:

$$\textbf{\% Cell Viability} = (OD_{sample} - OD_{blank} / OD_{control} - OD_{blank}) \text{ X } 100 \quad (2)$$

Statistical analysis

All the experiments were performed in triplicates. Results were expressed as the mean ± SD of values obtained in triplicate from three independent experiments.

RESULTS

Total Phenolic content and Antioxidant capacity

The results for total phenolic content, free radical scavenging activity and total antioxidant capacity were as shown in the Table 1.

Table 1 Total Phenolic Content, Free radical scavenging activity and total antioxidant capacity of *Nyctanthes arbortristis* plant extracts.

Treatment	Solvent systems	TPC* (mg GAE/ g)	FRS* (% DPPH Quenched)	TAA* (mg AAE/ g)
Elevated temperature	Water	44.09 ± 1.52	24.50 ± 3.680	42.48 ± 5.27
	Methanol	118.18 ± 4.30	58.74 ± 0.944	92.78 ± 8.72
	Water: Methanol	98.59 ± 2.25	52.98 ± 0.612	98.79 ± 9.54
	Chloroform: Methanol	106. 46 ± 2.21	62.64 ± 0.747	125.86± 16.05
25 °C	Water	33.41 ± 0.99	8.37 ± 1.347	35.03 ± 4.36
	Methanol	108.51 ± 2.32	56.96 ± 1.880	99.79 ± 13.53
	Water: Methanol	76.66 ± 1.45	42.44 ± 3.047	62.12 ± 4.85
	Chloroform: Methanol	131.94 ± 3.85	70.28 ± 3.227	118.49±16.90
Sonication	Water	36.09 ± 1.97	16.12 ± 3.602	45.96 ± 6.09
	Methanol	121.95 ± 4.73	45.18 ± 1.793	104.79 ±11.16
	Water: Methanol	81.36 ± 3.76	32.35 ± 4.490	86.08 ± 7.29
	Chloroform: Methanol	118.30 ± 3.27	45.12 ± 0.709	123.54± 13.64

**TPC- Total Phenolic Content, FRS- Free Radical Scavenging and TAC- Total Antioxidant Capacity. The TPC content was expressed as mg GAE/ g extract. The free radical scavenging activity was expressed as % DPPH scavenged. The total antioxidant activity was expressed as mg AAE/ g extract. Results were expressed in terms of Mean ± SD*

In the present study, the total phenolic content was estimated by the Folin-Ciocalteau method. The results obtained were as shown in Table 1. In the extracts prepared at elevated temperature, the total phenolic content ranged from 44.09 ± 1.52 to 118.18 ± 4.30 mg GAE/g of dry extract. The highest phenolic content was estimated in the methanolic extract whereas water extract had the lowest amount. In the extracts prepared at 25°C, the phenolic content ranged from 33.41 ± 0.99 to 131.94 ± 3.85 mg GAE/g of dry extract. Among the four solvent systems used, the chloroform: methanol extract had the highest amount of phenolics whereas water extracts had the lowest amount of phenolics. In the extracts prepared using sonication, the phenolic content ranged from 36.09 ± 1.97 to 121.95 ± 4.73 mg GAE/g of dry extract. The methanolic extract showed the highest amount of phenolics whereas water extracts showed lowest amount.

The antioxidant capacity of plant extracts were estimated by i) DPPH free radical scavenging capacity and ii) reduction of phosphomolybdenum reagent and the results were as shown in Table 1. For extracts prepared at elevated temperature, the % free radical scavenging activity ranged from 24 % to 62 %. For the extracts prepared at 25 °C, it ranged from 8 % to 70 %. For extracts prepared at sonication, it ranged from 16 % to 45 %.

The total antioxidant capacity estimated by the phosphomolydenum assay of the plant extracts was as shown in Table 1. For the extracts prepared at elevated temperature it ranged from 42.48 ± 5.27 to 125.86 ± 16.05 mg AAE/g of dry weight of extracts. For the extracts prepared at 25 °C, it ranged from 35.03 ± 4.36 to 118.49 ± 6.90 mg AAE/g of dry weight of extracts. The extracts prepared using sonication were in the range, 45.96 ± 6.09 to 123.54 ± 13.64 mg AAE/g of dry weight of extracts.

Antiproliferative activity

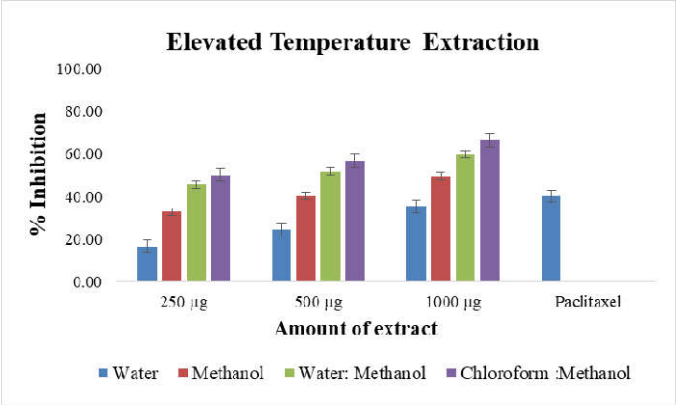

Figure 1% Cell growth inhibition of yeast cells treated with Nyctanthes arbortristis extracts prepared at elevated temperature after 24 hours

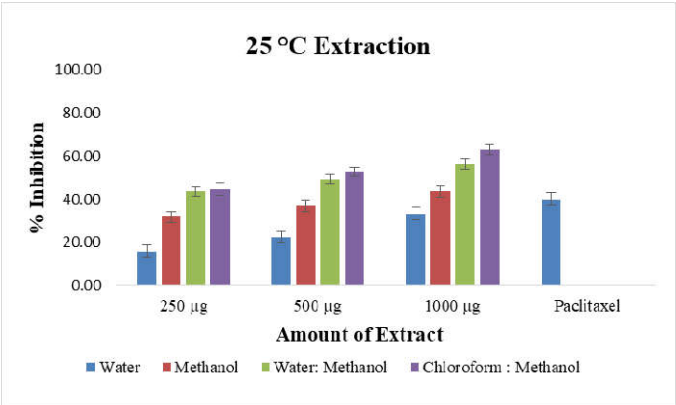

Figure 2 % Cell growth inhibition of yeast cells treated with Nyctanthes arbortristis extracts prepared at 25 °C temperature after 24 hours

The crude extracts of *Nyctanthes arbortristis* prepared by different extraction methods and solvents were tested for their antiproliferative activity on fission yeast *S. pombe*. Figure 1, 2 and 3 represent the percentage cell growth inhibition of crude extracts at different doses. It was clearly observed that the decrease in the growth of yeast cells was dose dependent. Moreover, the growth inhibition capacity of the extracts was compared with that of the standard drug Paclitaxel used in the treatment of lung cancer.

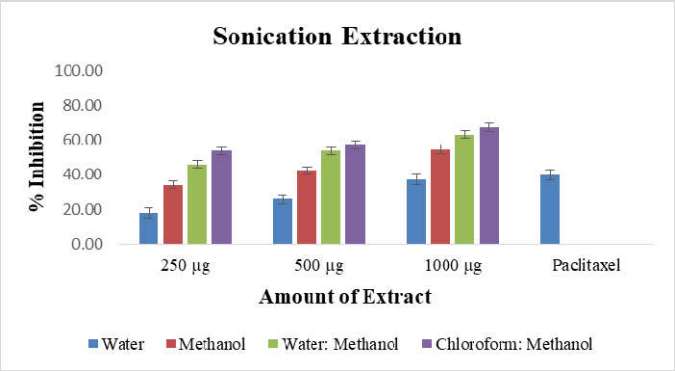

Figure 3 % Cell growth inhibition of yeast cells treated with Nyctanthes arbortristis extracts prepared using sonication after 24 hours

The cell viability of cells treated with Paclitaxel at 2.5 nm concentration was 60 % which means that Paclitaxel was capable of inhibiting growth of yeast cells upto 40%. Thus, in our study, crude extracts showing inhibition higher than 40 % were considered to have potential cytotoxic property. The antiproliferative activity of extracts prepared at elevated temperature ranged from 17 % to 66 % (Fig.1) after incubation of 24 hours. The antiproliferative activity of extracts prepared at 25 °C temperature ranged from 15 % to 63 % (Fig.2). As shown in Fig.3, the antiproliferative activity of extracts prepared at elevated temperature ranged from 18 % to 67 %. Among the four solvent systems used for extraction, it was observed that the chloroform: methanol extracts showed highest growth inhibition whereas water extracts showed lowest inhibition. From the graphs it was clearly observed that the order of growth inhibition of yeast cells was as follows: chloroform: methanol > water: methanol > methanol > water. Out of the three treatment methods used for extraction, sonication showed highest inhibition followed by elevated extraction and lowest was observed in cold extraction. Furthermore, in our study, it was observed that the water extracts showed growth inhibition less than 40 % whereas the remaining extracts showed inhibition higher than 40 %. Thus, it can be inferred that the water extracts had least potential for antiproliferative activity.

DISCUSSION

In the present study, our aim was to perform preliminary phytochemical analysis and antiproliferative activity of extracts of *Nyctanthes arbortristis*. As described earlier, this plant has several medicinal uses and has been called an herbal panacea (Meshram *et al*., 2012). In the present study, the crude extracts of the leaf of the plant were prepared using three different treatment conditions (Elevated and 25°C temperature and ultrasound waves) and four different solvents (Water, Methanol, Water: Methanol and Chloroform: Methanol) which provided us with a total of twelve extracts. The extracts were then quantified for their total phenolic content and antioxidant capacity. From the results, it was clearly observed that among the four solvent systems used for extraction, the highest total phenolic content was observed in Methanol and Chloroform: Methanol extracts whereas water extract had lowest amount of phenolics. In one study, the flower, stem, root and leaves of *Nyctanthes arbortristis* were evaluated for their total phenolic content in extracts prepared using different solvents. It was observed that the methanolic leaf extract had highest phenolic content whereas aqueous extracts had lowest amounts (Jaiswal and Thakur 2017). This is in agreement with the results obtained from our study. The significant differences in the phenolic content of the extracts may be attributed to different solvents used. This study shows that methanol and chloroform: methanol solvent system has yielded maximum phenolic content.

In the human body, oxidative stress is generated due to an imbalance between free radical production and antioxidant defences. This oxidative stress causes damage to biomolecules like lipids, proteins and nucleic acids. Antioxidants are stable molecules that neutralize free radicals by donating an electron and thus reducing the damage caused by these free radicals (Lobo *et al*., 2010). It is well known that oxidative stress has implications in pathogenesis of various diseases like cardiovascular, neurodegenerative, cancer and aging among others (Szymanska *et al*., 2016). Plants are a great source of natural antioxidants due to presence of secondary metabolites such as phenolic and flavonoid compounds which possess strong free radical scavenging property (Abdel-lateif *et al*., 2016).

In this study, antioxidant capacity of the extracts was estimated by the using DPPH scavenging capacity and Phosphomolybdenum assay. DPPH is a stable free radical and loses it deep purple colour and turns to yellow upon reaction with any oxidising compound. It is a rapid, simple, and widely used method to measure the ability of compounds to act as free radical scavengers or hydrogen donors (Kedare and Singh, 2011). In the present study, the highest free radical scavenging activity was observed in chloroform: methanol and methanol extracts whereas water extract had the lowest free radical scavenging activity. In a study by Rathee *et al*., 2007, methanolic fraction of ethyl acetate extract of leaf demonstrated about 23 % DPPH scavenging activity. Another study showed free radical scavenging activity of a methanol extract to be 27.8 % (Kumari *et al*., 2012). In our study, we observed that the DDPH scavenging activity of methanol extract by three methods ranged from 45 % to 59 %. This variation in free radical scavenging activity is probably because extraction was performed using different treatment methods.

Phosphomolybdenum assay is a quantitative method used for evaluation of the antioxidant capacity indicated by electron donating capacity (Prieto *et al*., 1999). The phosphomolybdenum method is quantitative since the total antioxidant activity of the extracts is expressed as the number of equivalents of ascorbic acid. Again, the highest antioxidant activity was observed in the methanol and chloroform: methanol extracts and lowest was observed in water extracts. In a study performed by Michael *et al*., 2013, strong antioxidant activity was observed in methanolic extracts of leaf. To the best of our knowledge, the antioxidant capacity of water, water: methanol and chloroform: methanol extracts of leaf has not been reported.

Several reports are available where it has been demonstrated that phenolic compounds present in the plant extracts has a major contribution to antioxidant activity (Cai *et al*., 2004; Li *et al*., 2008; Sun *et al*., 2002; Zou *et al*., 2011). Similar phenomena were observed in our study as well. Here, the highest phenolic content was observed inmethanol and chloroform: methanol and the corresponding antioxidant and free radical scavenging activities were also highest among all the extracts. Thus, it could be implied that the phenolics

present in the crude extract contribute to the antioxidant capacities of *Nyctanthes arbortristis*.

Further, the crude extracts were screened for its antiproliferative activity using *Schizosaccharomyces pombe*. Here, *S. pombe* was selected for performing a pilot screen to evaluate the growth inhibitory potential of the extracts. Previously, a bioactive compound known as 2', 4' – dihydroxychalcone from leaves of *Corema album* was identified using *S. pombe* (Sánchez-Picó *et al*., 2014). In another study, the mechanism of action of a known phytochemical Plumbagin was studied using *S. pombe* (Lee *et al*., 2012). Additionally, cytotoxic effect of various extracts from *Phyllanthus emblica* L. were screened using S. pombe (Desai and Braganza, 2016). Based on these studies, we tested the cytotoxic potential of our extracts on *S. pombe*. From this screening, we identified that the methanol, water: methanol and chloroform: methanol extracts of all three methods showed higher growth inhibition as compared to Paclitaxel (a chemotherapeutic drug used for lung cancer treatment). Paclitaxel is a plant based anticancer drug isolated from the bark of the Pacific Yew, *Taxus brevifolia* Nutt (Sultana *et al*., 2014). The mechanism of action of Paclitaxel involves interaction with polymerized tubulin and to promote the formation of microtubules and prevents their disassembly. Due to this the cells are arrested in G2-M phase of the cell cycle (Georgiadis *et al*., 1997). This pre-screen concludes that the methanol, water: methanol and chloroform: methanol extracts have high growth inhibitory potential and can be further screened using *in vitro* and *in vivo* techniques for identification of bioactive component against lung cancer from this plant.

CONCLUSIONS

From the present study, it can be concluded that the *Nyctanthes arbortristis* plant extracts possess high content of phenolics and has strong antioxidant property. Furthermore, it can be concluded that the methanol, water: methanol and chloroform: methanol extracts of the plant possess cytotoxic effect on yeast cells. This suggests that further screening of extracts on lung cancer cell line could provide us with new bioactive molecules against lung cancer.

Conflict of interest

There are no conflicts to declare.

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