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Characterization and anti-proliferative activity of acetone extract and its hexane fraction from *Wedelia trilobata* leaves

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Abstract
The current study emphasizes on the utilization of the invasive allelopathic plant, *Wedelia trilobata* for its medicinal effect. It provides an elaborative phytochemical analysis of acetone extract (NALTW-1) and its hexane fraction (NALTW-H1) of *Wedelia trilobata* leaves, prepared by accelerated solvent extraction technique. The samples (NALTW-1 and NALTW-H1) were evaluated for their antioxidant potential in terms of2, 2´-azinobis[3-ethylbenzthiazoline]-6-sulfonic acid (ABTS) and 2,2-diphenyl-1-picrylhydrazyl(DPPH) assays and were also subjected to qualitative phytochemical analysis. They were characterized by high performance liquid chromatography (HPTLC) for quantifying antioxidant compounds (ascorbic acid, gallic acid, rutin and quercetin) and nucleobases (adenine, cytosine, guanosine, thymine and uracil). The results of the phytochemical analyses: ABTS and DPPH assays revealed substantial antioxidant potential of the samples. Considering these results, the samples were further evaluated for their anti-proliferative activity against the breast carcinoma cell line, MCF-7 using 3-(4,5-dimethylthiazol-2- yl)-2,5-diphenyltetrazolium (MTT) assay. *In vitro*, NALTW-1 and NALTW-H1 demonstrated moderate anti-proliferative activity against MCF-7 cells with IC50 values 570.78 µg/ml and 44.50.98 µg/ml, respectively. Thus, this study highlighted therapeutic benefit of the invasive plant, *Wedelia trilobata*.

Keywords
Allelopathy, Antioxidant, HPTLC, Nucleobase, Phytochemical analysis, *Wedelia trilobata*.

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Introduction

Medicinal plants have always been an advantage to the economy. They play a pivotal role in traditional as well as modern medicine. This is attributable to the numerous bioactive principles such as alkaloids, terpenoids and polyphenolic compounds including flavonoids which are characteristic to them (Sharma and Devkota, 2015). However, there are some medicinal plants which pose ecological threats even though they possess various medicinal and pharmacological benefits. To begin with, such herbs, the invasive plants is one of such kind. These are often menace to their neighboring plants as they are allelopathic and are known to suppress growth of other plant species via release of phytotoxic allelochemicals through leaf extracts, volatile organic compounds and root exudates (Dai et al., 2016). An example of such an invasive plant is *Wedelia trilobata* (*W. trilobata*) which has a vast array of medicinal properties and has great ethno medical significance in traditional medicine (Balekar *et al.*, 2012). It is a storehouse of numerous bioactive compounds such as flavonoids, saponins, tannins and terpenoids. Such biochemical composition has imparted *W. trilobata* with medicinal properties, namely analgesic, anti-diabetic, anti-inflammatory, anti-microbial, antitumor, hepatoprotective, larvicidal, uterine contraction and wound healing(Balekar *et al*., 2012; Govindappa and Poojashri, 2011). This perennial herb of Asteraceae family is native to Central America and has been introduced as an ornamental plant or invaded many geographical (tropical and sub-tropical) regions including the Indian sub-continent (Qi *et al*., 2014). It is considered as a threat to the neighboring native flora in these habitats being an invasive allelopathic plant (Dai *et al*., 2016). Therefore, there is an urgent need to control such invasive allelopathic plants. One of the ways could be exploiting such plants for their benefits to mankind.

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Considering the above facts, in the present study, acetone extract (NALTW-1) and its hexane fraction (NALTW-H1) prepared from the leaves of *W. trilobata* were investigated for their potential anti-proliferative activity against the breast carcinoma cell line MCF-7 and were characterized for bioactive compounds namely antioxidant compounds (ascorbic acid, gallic acid, rutin and quercetin) and nucleobases (adenine, cytosine, guanosine, thymine and uracil) that are known to possess several biological activities (Bhardwaj *et al*., 2015; Mishra *et al*., 2017) by high performance thin layer chromatography (HPTLC). Antioxidant activity was evaluated by 2, 2´-azinobis[3-ethylbenzthiazoline]-6-sulfonic acid (ABTS) and 2,2-diphenyl-1-picrylhydrazyl(DPPH) assays and; total flavonoids and total phenolic contents were also determined. In conclusion, the therapeutic usage of an invasive plant such as *W. trilobata* was evaluated which could benefit the pharmaceutical industries besides enabling its management.

Materials and Methods

Chemicals andReagents

All the standards and reagents used were of analytical grade and were procured from Merck (USA), Sigma-Aldrich (USA), S.D. fine Chemical Ltd. (Mumbai, India) and Sisco Research Laboratories Pvt. Ltd. (Mumbai, India). Water used throughout was of Milli-Q grade.

Plant Material

W. trilobata (Tridax), plant was grown in the lawns of Naval Materials Research Laboratory (NMRL), Ambernath, Maharashtra, India. The plants were used to collect around 1000 kg leaves (fleshy, hairy, 4–9cm long and 2–5cm wide, serrate or irregularly toothed, normally with pairs of lateral lobes, and dark green above and lighter green below). The leaves were washed, shade dried at room temperature (25-30ºC) and pulverized into a fine powder to obtain 180 kgdry powder which was further used for extraction.

Instruments

Other than maceration, the accelerated solvent extraction (ASE 350) system equipped with a solvent controller unit from Dionex Corporation (Sunnyvale, CA, USA) was used to prepare the crude acetone extract of *W. trilobata*. The ASE system was also employed for the preparation of hexane fraction of the ASE extracted crude acetone extract. The maceration and ASE generated extracts and; the hexane fraction of the ASE generated acetone extract were freeze dried under vacuum in an Allied Frost 5 lyophilizer. HPTLC analysis was performed using a system from CAMAG, Switzerland. The system consisted of a Linomat 5 applicator for sample application; Reprostar 3 for documentation and; Scanner 3 for densitometric scanning. The absorbances for ABTS, DPPH assayand determination of total flavonoid and total phenolic contents were measured using BIO-RAD Model1680, microplate reader.

Extraction and Fractionation

Preparation of acetone extract

A known weight of the pulverized leaves powder was extracted with acetone. Two extraction methods were employed: maceration and accelerated solvent extraction (ASE) techniques. During maceration, the leaves powder was kept on a rotary shaker for 48h at room temperature (RT) in the acetone (solvent). Solvent was decanted every 48h and fresh solvent was added. This was repeated until the solvent extract became colorless. All the solvent extracts were then pooled and concentrated under vacuum until dry viscous product was collected and was designated as crude acetone extract (NALTW-M1). Whereas, the extraction procedure using ASE system was carried under optimized conditions of high pressure (100 bars) and temperature (50ºC) and a ratio of 1:1 was used for the leaves powder and sand in the extraction cell to enhance solvent mixing. The percentage yields of the crude extracts prepared by both maceration and ASE technique were calculated. The yield was higher for the crude extract prepared by ASE (NALTW-1) and therefore, was further taken up for fractionation using the hexane as solvent.

Preparation of hexane fraction

The crude acetone extract yielded by ASE (NALTW-1) was further fractionated with hexane. The acetone extract (100g) was suspended in hexane (200ml) and the mixture was stirred magnetically at RT for 5h. Hexane layer (180ml) was separated and fresh hexane (200ml) was added to the residue. The mixture was stirred mechanically for 3hat room temperature. This was continued till the hexane layer became almost colorless. The combined hexane layer was dried over anhydrous sodium sulfate and evaporated in vacuuo using rotary evaporator to yield the hexane fraction (NALTW-H1) of the acetone extract as a dark viscous residue (75g, 75%).

Antioxidant Activity

The antioxidant potential of the crude acetone extract (NALTW-1) and its hexane fraction (NALTW-H1) were evaluated by ABTS and DPPH assays.

ABTS antioxidant assay

ABTS free radical scavenging activity was measured as described elsewhere (Chatatikun and Chiabchalard, 2013). The absorbance was measured at 734nm after 6 minutes and $ABTS^{\cdot+}$ scavenging activity was expressed as µM TE (Trolox equivalent)/ gram of sample. Trolox standard (10-1000 µM) was prepared in ethanol to produce a calibration curve.

DPPH antioxidant assay

DPPH assay was done according to the method described by Bhardwaj *et al*., 2016 with some modifications. The absorbance was read at 515nm and the standard curve was drawn in a range of 62.5-1000µM Trolox. The results were expressed as µM TE (Trolox equivalent)/ gram of sample.

Phytochemical Analysis

The phytochemical analysis of NALTW-1 and NALTW-H1 were carried out using various methods developed to detect alkaloids, anthraquinones, cellulose, flavonoids, glycosides, phenols, quinones, saponins, steroids, tannins and terpenoids, in accordance to the standard procedures described elsewhere (Ayoola *et al*., 2008; Raja and Sama, 2012).

High Performance Thin Layer Chromatography (HPTLC) Analysis

The samples, NALTW-1 and NALTW-H1 were characterized by HPTLC for antioxidant compounds and nucleobases.

HPTLC for antioxidant compounds

Identification and quantification of antioxidant compounds (ascorbic acid, gallic acid, rutin and quercetin) in NALTW-1 and NALTW-H1 was performed in a similar procedure as mentioned earlier (Bhardwaj *et al*., 2015). Stock solutions of the standards namely ascorbic acid (asc) gallic acid (gal), quercetin (que) and rutin (rut) were prepared by dissolving 1mg of each standard in 1ml of methanol. A mixture comprising equal volumes from each of these standards was prepared and was used for further analysis. Three different volumes (3, 4, 5µl) of the mixture solution containing antioxidant standards and 20µl of each sample (each at a concentration of 40mg/ml) were spotted on a 20 x 20cm glass backed silica gel 60 F_{254} HPTLC plate (Merck). The mobile phase used constituted of ethyl acetate: dichloromethane: formic acid: glacial acetic acid: methanol (10:10:1:1:2 v/v). The standard antioxidant compounds detected in NALTW-H1 and NALTW-H1 were also quantified densitometrically. The developed plates were derivatized using alcoholic DPPH reagent (20%) and also by aluminium chloride solution(1%) for confirming the presence of antioxidant compounds. Theses plates were then documented under white light reflectance (White R) and 366nm, respectively.

HPTLC analysis for nucleobases

The nucleobases adenine (aden), cytosine (cyt), guanosine (guanos), thymine (thy) and uracil (ura) were detected and thus, quantified by the HPTLC method described elsewhere (Mishra *et al*., 2017)in the samples (NALTW-1 and NALTW-H1). Prior to the analysis stock solutions of the nucleobase standards were prepared by dissolving 0.5mg of each standard in 1 ml of methanol. A mixture comprising equal volumes from each of these standards was prepared and used for further analysis. The samples were used at a concentration of 40mg/ml. Twenty microliter of each sample (NALTW-1 and NALTW-H1) solution was applied as 6mm wide bands, using a CAMAG Linomat 5 sample applicator equipped with a 100µl syringe (Hamilton) on a
20 x10cm glass backed silica gel 60 F_{254} HPTLC plate (Merck). The standard mixture solution was applied at three different volumes (3, 4, 5µl) on the same plate. The plate was then developed at RT in a CAMAG twin-trough vertical development chamber using the mobile phase; dichloromethane: methanol: formic acid (8:2.25:0.8 v/v/v). Following this, densitometric scanning was executed at 254nm using CAMAG TLC Scanner 3 equipped with win Cats software up to a migration distance of 85mm. For quantification, the area of the peaks corresponding to the R_f (retardation factor) value of each respective nucleobase were recorded and the amount present was calculated via the regression equation obtained from the calibration plot.

***In-vitro* anti-proliferative activity**

Cell lines and maintenance

Cell culture reagents, media, antibiotics and other supplements were purchased from Sigma-Aldrich, USA. Fetal Bovine Serum (FBS, Certified) was procured from Invitrogen Life Sciences, USA. Breast Cancer cell line (MCF-7), was maintained in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich, USA) with 10% FBS, 100 units/ml penicillin and 100 µg/ml streptomycin (Invitrogen Life Sciences, USA). The cells were maintained at 37°C in a humidified atmosphere with 5% CO_2. All cell culture work was performed under aseptic conditions inside a laminar airflow hood.

Treatments and MTT assay

The anti-proliferative activity was determined by the MTT assay according to the method described elsewhere with some modifications (Al-Oqail *et al*., 2015). Eight thousand MCF-7 cells were plated in each well of 96-well tissue culture plates in 100 µl of culture medium supplemented with 10% FBS. The cells were allowed to attach for 24h and then were treated with 100, 50, 25, 12.5, 6.2 5µg/ml each of NALTW-1 and NALTW-H1 (prepared as stock of 10mg/ml in DMSO) for 24h by adding 100 µl of medium containing required amounts of NALTW-1 and NALTW-H1. Three hours of the stipulated time, 20 µl of 5 mg/ml MTT (Sigma Aldrich, USA) solution was added to each well and incubated further for 3h to allow formation of formazan crystals. The culture medium was completely aspirated by applying vacuum suction and 200 µl of DMSO was then added in each well to dissolve the crystals. The intensity of the purple color produced was estimated by determining the absorbance at 550nm against DMSO blank with the help of a spectrophotometer (BIO- RAD Model1680, microplate reader). All the treatments were done in triplicates and the results were represented as dose- response curves.

Results and Discussion

Extraction & Fractionation

The accelerated solvent extraction (ASE) technique has advantages of minimum solvent usage; extracts polar compounds (Bhardwaj *et al*., 2015; Kukula-Koch *et al*., 2016) and is more efficient extraction technique compared to maceration (Kukula-Koch *et al*., 2016; Nayak *et al*., 2015). In the study, ASE proved to be a faster technique with lesser use of the solvent, acetone. The maceration process yielded about 4.5-5.0 % of the acetone extract (NALTW-2) whereas, ASE yielded about 6.0-8.0 % of the acetone extract (NALTW-1).

Since, the percentage yield of the acetone extract obtained from ASE (i.e. NALTW-1) was higher than maceration (NALTW-2), it was considered for further fractionation. The percentage (%) yield of NALTW-1 and NALTW-2 are given in Table 1.

Table 1: Percentage yield of *W. trilobata* leaves acetone extracts.

EXTRACT	YIELD (%)
NALTW-1	4.5-5.0
NALTW-2	6.0-8.0

Fractionation of NALTW-1 was carried out with hexane using ASE technique. The percentage yield obtained for the hexane fraction, NALTW-H1 was 77.24%. NALTW-1 was further characterized and evaluated for anti-proliferative activity against the breast carcinoma cell line MCF-7 along with its hexane fraction (NALTW-H1).

ABTS and DPPH Assays

ABTS and DPPH assays are frequently employed for determining the antioxidant potential of the plant extracts and antioxidant compounds (Gutiérrez *et al*., 2014). In the present study, NALTW-H1 had relatively higher ABTS radical scavenging activity than NALTW-1; whereas, the antioxidant capacity measured by DPPH assay was higher for NALTW-1. The results are summarized in the Table 2. The plausible reason for the absence of correlation between the results of the ABTS and DPPH assays could be the variation in the oxidation kinetics of the same antioxidant compound(s) (e.g. polyphenols) during these assays, although these two employ the same single electron transfer mechanism (Boulanouar *et al*., 2013). Overall, it could be inferred that both the samples of *W. trilobata* (NALTW-1 and NALTW-H1) exhibited substantial antioxidant activity contributed by various antioxidant compounds such as polyphenols.

Table 2: Antioxidant potential of acetone extract (NALTW-1) and the hexane fraction (NALTW-H1) prepared from *W. trilobata* leaves determined in terms of ABTS and DPPH assays. ABTS radical scavenging activity is expressed as micromole Trolox equivalent per gram of the sample (mM TE/g of sample). DPPH radical scavenging activity is expressed as micromole Trolox equivalent per gram of the sample (mM TE/g of sample). The values are expressed in triplicates as Mean±S.D.

Sample	ABTS radical scavenging activity (µM TE / g of sample)	DPPH radical scavenging activity (µM TE / g of sample)
NALTW-1	330.88±0.00	1314.78±20.00
NALTW-H1	466.32±0.21	1114.12±2.21

Phytochemical Analysis

The phytochemicals are secondary metabolites that contribute majorly towards the biological activities of medicinal plants (Yadav *et al*., 2014). The results of the phytochemical analysis of *W. trilobata* acetone extract (NALTW-1) and its hexane fraction (NALTW-H1)are given in Table3.

It could be concluded from these results that the saponins and cellulose were not detected in either of the samples. Whereas, alkaloids, anthraquinones and quinones were present in the acetone extract (NALTW-1) only and were absent in the hexane fraction (NALTW-H1). Alkaloids are the basic natural products which are regarded as the most efficient therapeutic agents among the plant metabolites. Their prime medicinal properties include analgesic and anti-bacterial activities (Pradeep *et al*., 2014). On the other hand, the anthraquinones are considered as one of the most potent agents in metastatic breast cancer (Thamaraiselvi and Jayanthi, 2012).

The phytochemicals namely, flavonoids, glycosides, phenols, steroids, tannins and terpenoids were detected in both samples of *W. trilobata* leaves. The flavonoids and phenolic compounds in plants have been reported to exert multiple biological effects including antioxidant, freeradical scavenging abilities, anti-inflammatory, anticarcinogenic etc. (Thamaraiselvi and Jayanthi, 2012).

Table 3: Phytochemical analysis of the crude acetone extract (NALTW-1) and the hexane fraction (NALTW-H1) prepared from *W. trilobata* leaves.

Phytoconstituent	NALTW-1	NALTW-H1
Flavonoids	+	+
Phenols	+	+
Tannins	+	+
Saponins	-	-
Steroids	+	+
Terpenoids	+	+
Cellulose	-	-
Glycosides	+	+
Quinones	+	-
Anthraquinones	+	-
Alkaloids	+	-

"+" = present; "-" = absent

Steroids are known for their cholesterol reducing properties and also regulate immune response (Pradeep *et al*., 2014).

Tannins exhibit potent anti-microbial (Thamaraiselvi and Jayanthi, 2012) and wound healing properties (Pradeep *et al.*, 2014). Terpenoids are a class of natural isoprenoids (Thamaraiselvi and Jayanthi, 2012) which possess a wide range of bioactivities including antihyperglcemic, anti-allergic, anti-inflammatory, anti-microbial, anti-viral and immunomodulatory activity (Pradeep *et al*., 2014). Thus, the samples were rich in various bioactive principles as revealed bythe qualitative phytochemical analysis.

High Performance Thin Layer Chromatography (HPTLC) Analysis

HPTLC for antioxidant compounds

The HPTLC analysis of antioxidant compounds demonstrated presence of rutin and quercetin in both NALTW-1 and NALTW-H1. NALTW-1 additionally contained ascorbic acid. Quercetin is a plant flavonol with marked pharmacological health benefits. It is a potent reducing agent which along with other dietary reductants such as ascorbic acid (vitamin C) protect body against oxidative stress (Jan *et al*., 2010). However, beyond its antioxidant activity, quercetin exhibits several other biological effects such as anti-carcinogenic, anti-inflammatory, anti-viral, cardioprotective, muscle-relaxing and neuroprotective properties (Davis *et al*., 2009; Jan *et al*., 2010). Rutin (quercetin-3-O-rutinoside) is another polyphenolic bioflavonoid which shows a wide range of biological activities due to its significant antioxidant properties. Usually, it is used for its anti-allergic, anti-cancer, anti-inflammatory, anti-microbial and anti-viral properties. It has also shown protective role in liver diseases, cataracts and cardiovascular diseases (Davis *et al*., 2009; Seif, 2012). The quantities of these antioxidant compounds (ascorbic acid, rutin and quercetin) detected in the samples are shown in Table 4. The HPTLC profiling for the antioxidant compounds at 254 nm is given in Figure 1.

Table 4: Quantities of antioxidants occurring in crude acetone extract (NALTW-1) and the hexane fraction (NALTW-H1) of *W. trilobata* leaves.

Antioxidant (µg antioxidant/ mg of sample)	Samples	
	NALTW-1	NALTW-H1
Quercetin	0.25	1.32
Rutin	0.30	0.80
Ascorbic acid	0.16	-

Post derivatization with DPPH revealed presence of distinct yellow zones under white illumination on the entire surface of each track of the derivatized plate where the samples NALTW-1 and NALTW-H1 had ascended (Figure2a). These yellow zones substantiated the high content of compounds capable of scavenging DPPH free radical, thus, elucidating the appreciable antioxidant potential of these samples (Misra *et al*.,2012).

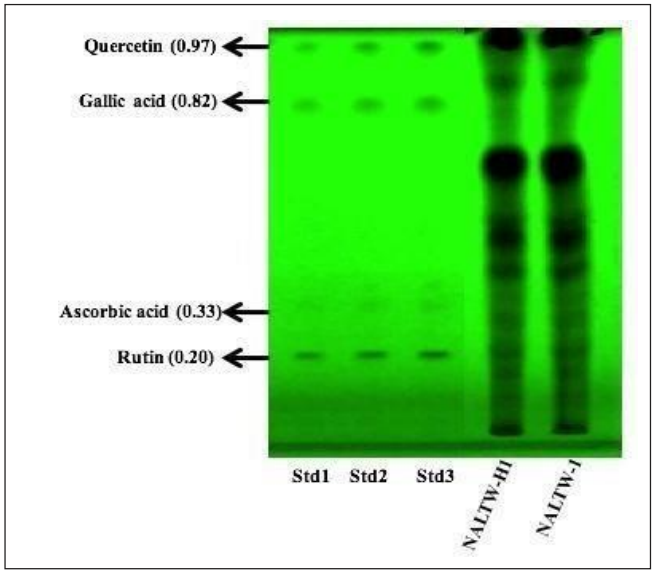

Figure 1.HPTLC profiling for detecting the antioxidant compounds: ascorbic acid, gallic acid, rutin and quercetin) in crude acetone extract (NALTW-1) and the hexane fraction (NALTW-H1) of *W. trilobata*.

*Values in parentheses indicate the R_f (retardation factor) values of respective standard antioxidant compound.

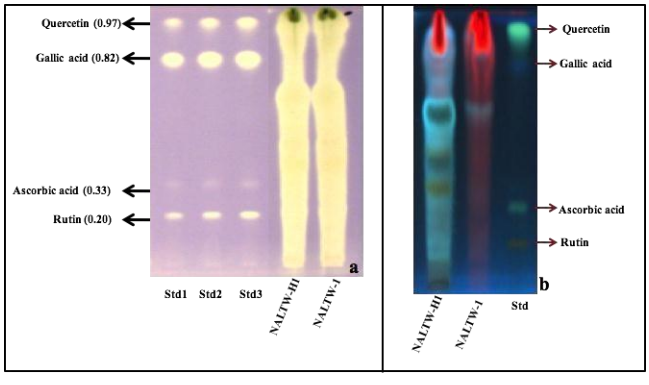

Figure 2. HPTLC plates derivatized with DPPH under white light illumination (a) and aluminium chloride at 366 nm (b) to detect antioxidant compounds in the acetone extract (NALTW-1) and the hexane fraction (NALTW-H1) of *W.trilobata* leaves.

After derivatization of the developed plate with aluminium chloride at 366nm, characteristic colored bands corresponding to antioxidants were visible (Figure 2b). This verified the richness of the NALTW-1 and NALTW-H1 with respect to antioxidant compounds.

HPTLC analysis for nucleobases

HPTLC characterization of the samples (NALTW-1 and NALTW-H1) revealed that only adenine was quantifiable in NALTW-H1. The quantity of adenine in NALTW-H1 was calculated to be 0.26µg/mg of fraction, whereas, NALTW-1 was not found to contain significant amount of any standard nucleobase tested. Clifford (1981), demonstrated that the

purine, adenine is an important precursor of nucleic acid synthesis in the intestinal cells (Savaiano and Clifford, 1981). Besides, adenine also aids in cellular respiration (Mishra *et al*., 2017). Figure 3displays HPTLC profile for the presence of nucleobases in the samples, NALTW-1 and NALTW-H1.

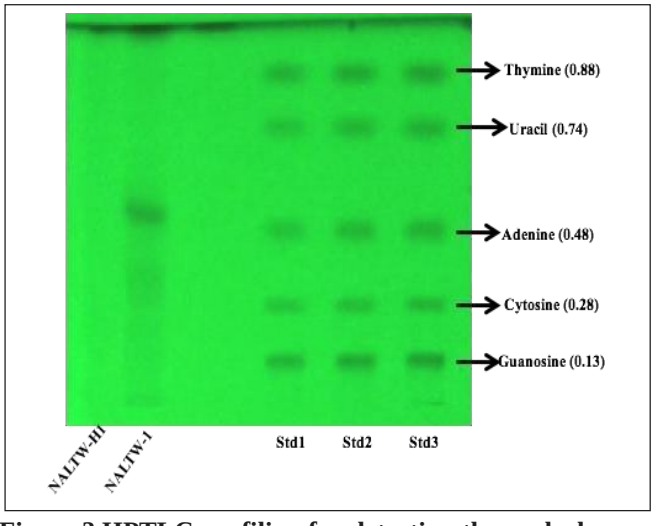

Figure 3.HPTLC profiling for detecting the nucleobases: adenine (aden), cytosine (cyt), guanosine (guanos), thymine (thy) and uracil (ura) in crude acetone extract (NALTW-1) and the hexane fraction (NALTW-H1) of *W. trilobata* leaves.

*Values in parentheses indicate the R_f (retardation factor) values of respective standard nucleobase.

***In vitro* anti-proliferative activity**

The acetone extract (IC50= 570.78 µg/ml) and its hexane fraction (IC50= 44.50.98 µg/ml) prepared from *W. trilobata*, were found to show moderate anti-proliferative activity. These might inhibit tumor cell proliferation and induce significant cell death. The in vitro anti-proliferative activity of NALTW-1 and NALTW-H1 was evaluated against the breast carcinoma cell line MCF-7 using the MTT metabolic assay. The inhibitory activity (IC_{50}; 50% inhibitory concentration) of the above-mentioned samples on cell proliferation was determined. As shown in Figure 4, NALTW-1 and NALTW-H1 were found to show moderate anti-proliferative activity having IC_{50} value of the order of 570.78 µg/ml and 44.50.98 µg/ml respectively. Thus, the acetone extract (NALTW-1) had better anti-proliferative activity than its hexane fraction (NALTW-H1).

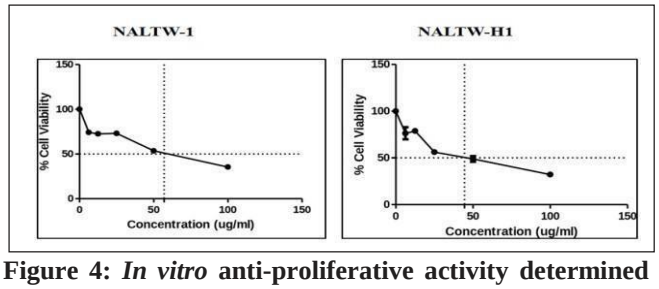

Figure 4: *In vitro* anti-proliferative activity determined via MTT assay of *W. trilobata* leaves acetone extract (NALTW-1) and its hexane fraction (NALTW-H1).

This could be attributable to a relatively higher concentration of ascorbic acid, quercetin and rutin in NALTW-H1 and higher ABTS scavenging activity than NALTW-1. As mentioned earlier the antioxidant compounds: ascorbic acid, quercetin and rutin (Jan *et al*., 2010; Davis *et al*., 2009; Seif, 2012; Sharma *et al*., 2013) possess anti-cancer properties. In conclusion, the *W. trilobata* acetone extract (NALTW-1) and hexane fraction (NALTW-H1) might possibly inhibit tumor cell proliferation and induce significant cell death.

Conclusion

The current study illustrated that the best plausible way to manage an invasive allelopathic plant such as *W. trilobata* that often poses threat to its nearby plants could be by exploiting its medicinal benefits for human welfare. During the study, the acetone extract (NALTW-1) and its hexane fraction (NALTW-H1) of *W. trilobata* leaves demonstrated presence of major antioxidant compounds such as rutin and quercetin which could have contributed towards their anti-proliferative activities against the breast carcinoma cell line, MCF-7, thereby, supporting its anti-proliferative activity.

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CONFLICT OF INTEREST: None

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