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Investigation on the biological attributes of *Tragia involucrata* Linn. using *in vitro* methods

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Abstract
Medicinal plants are rich source of bioactive phytoconstituents used for treating and healing a variety of ailments. Five different extracts of the leaves of *Tragia involucrate* were evaluated for their anticancerous activity against K562 cell lines. The phytoconstituents were extracted with petroleum ether, chloroform, ethyl acetate and methanol by single solvent maceration with continuous shaking. Preliminary phytochemical analysis by Thin Layer Chromatography (TLC) revealed the presence of steroids, anthraquinones, phenolic compounds etc in the extracts. The antioxidant activity of extracts was determined by DPPH spraying on TLC plates. The haemagglutination potential and complement activation were performed using aqueous extract. It showed mild haemagglutination ability whereas it could not activate complement. The extracts were tested for cytotoxicity by MTT assay. The chloroform and ethyl acetate extracts showed the antiproliferative activity against K562 cell lines.

Keywords: *Tragia involucrata*, K562, TLC, PE extract, EA extract, MOH extract, CHF extract

1. Introduction
Phytochemical investigation is active throughout the world in search for new active plant secondary metabolites and for its valuable and diverse biological activities. Medicinal plants used in the traditional medicine provide a promising and largely unexplored, unidentified source for the creation and development of potentially new drugs [1]. Therefore, it is of great interest to carry out screening of these plants in order to validate their use in traditional medicine and also to explore the active principles by isolation and characterisation that contributes for the bioactivity. *Tragia involucrata* Linn. Is a perennial twinning herb commonly known as Indian stinging nettle seen growing in areas like tall grasslands, meadows, borders of forests and fields [2]. It is popular for various medicinal uses in the indigenous system of medicine. The leaves of *T. involucrata* are sweetish, tonic, carminative, antipyretic and emmenagougue. They are used as medicine to treat many different ailments. The most common use of leaves is to make tea that would be consumed to treat intestinal worms. There are also accounts of this plant being used to treat malaria and it is used for the fever, skin eruption, diabetes, venereal diseases and haemorrhoids. Root contains tannins, flavonoids, triterpenoids, saponins, alkaloids, phytosterols, phenolic compounds, catachins, proteins, lipids and aminoacids. Phytochemical screening have shown the presence of alkaloids, coumarins, flavonoids, glycosides, steroid glycosides, cardiac glycosides, saponins, tannins, and terpenoids in this plant [3].
The great traditional knowledge and the rich biodiversity in India provide the basis for many effective anticancer agents. The study of folk medicinal practices in Kerala revealed the ethno medical use of the aerial parts of *Tragia involucrata* along with other medicinal plants to treat certain tumours in few villages in Kerala, India [4]. This plant is aknown antitumor agent in ancient system of medicines such as Ayurveda, Sidha, and Unani. The anti-microbial, anti-inflammatory and antifertility activity of *T. involucrata* has been reported [5, 6, 7]. Recently Joshy *et al*., 2011 have demonstrated the potent *in vitro* anticancer activity of *T. involucrata* extracts against cancer cells [7]. The preliminary phytochemical screening results revealed the presence of alkaloids, carbohydrates, protein, tannins, flavonoids, sterols and saponins in the different extracts of *T. Involucrata* [8]. Several colorless phytocompounds have been isolated and characterized from *T. involucrata* such as vinyl hexylether, shellsol, 2,4-dimethyl hexane, 2-methylnanone, and 2,6-dimethyl heptanes [9].
The present study is mainly focussed to screen the preliminary phytochemicals using different spraying reagents after thin layer chromatographic separation.

The antioxidant property of the extracts was also screened by DPPH spray. The *in vitro* cytotoxic potential of the plant was evaluated against K562 cell line by MTT assay. Further, haemagluttination, protein estimation, and complement activation potentials were also investigated.

2. Materials and Methods

2.1 Plant extracts preparation

Fresh leaves of *Tragia involucrata* were collected from Calicut University campus, Tenjipalam, Malappuram district, Kerala, India. Taxonomic authentication of the plant was done by Dr. A.K. Pradeep, Angiosperm taxonomy division, Department of Botany, University of Calicut. The leaves were shade dried and powdered using a blender. The single solvent extraction method was used for the preparation of different extracts. Powdered leaf was soaked separately in different solvents such as petroleum ether, chloroform, ethyl acetate and methanol.

2.2 TLC based identification of secondary metabolites

TLC silica gel 60 F_{254} aluminium sheets of analytical chromatography grade were used for the experiment. 5 µl of each extract were diluted in respective solvents and spotted at 3 places in TLC sheet. The plates were allowed to air dry and then kept in the developing chamber saturated with different solvent combinations; petroleum ether: acetone (3:2), petroleum ether: ethyl acetate (6:4), chloroform: methanol (3:2) and allowed to run for about 15 minutes. The solvent combination which showed maximum separation was selected for the identification of the phytoconstituents. The plates were taken out of the chamber, the solvent front was marked and then air dried. The R_f value of the bands under visible, short UV light 254nm and long UV light 365nm were calculated. After the completion of chromatographic run with suitable solvents, the plates were air dried and different spraying reagents were applied. Steroids, sugars, terpenoids were detected using anisaldehyde-sulphuric acid reagent. Phenolic compounds were detected using Fast Blue Salt reagent (FBS). Iodine reagent was used to detect compounds having conjugated double bond and potassium hydroxide reagent was used for the detection of anthrones, anthroquinones and coumarins.

2.3 Antioxidant activity using DPPH

After the development of TLC plates, 0.2% of DPPH solution (2,2-diphenyl-1-picrylhydrazyl) in methanol was sprayed on the surface of developed TLC plates and incubated in the dark for 10 min at room temperature.

2.4 Bradford protein assay

Bradford protein assay was used to measure the concentration of total protein in the aqueous extract. BSA (1mg/ml) was used as the protein standard. Five dilutions of the protein standard were prepared with a concentration of 5-30µg of protein and the final volume in each tube was made up to 100µl with 1XPBS. The tube containing 100µl of PBS alone served as negative control. Unknown sample was prepared by diluting 10µl of each sample to 100µl with 1XPBS. 1ml of Bradford reagent was added to each tube mixed well and incubated in room temperature for 5 minutes and absorbance measured at 595 nm. From the standard graph, concentration of unknown sample was calculated.

2.5 Haemagglutination assay

Human peripheral blood was collected from healthy volunteers, diluted in 2 volumes of PBS and centrifuged at 3000 rpm for 10 minutes. Pellet was then washed in PBS until the supernatant becomes clear. 200µl of the pellet was made up to 10 ml, to make 2% erythrocyte suspension. 96 well U bottom microtiter plates were used for the haemagglutination assay. The crude aqueous extract was serially diluted in PBS and incubated with an equal volume of 2% erythrocyte suspension at room temperature for 30 minutes. The wells that develop mat like appearance indicates positive agglutination and those with red button of erythrocytes indicate negative agglutination. Inverse of the highest dilution of the extract that shows visible agglutination is the titre value and is regarded as 1 haemagglutination unit (HU). PBS diluted with erythrocyte alone served as negative control whereas serially diluted anti-serum treated with erythrocyte suspension made the positive control. Specific activity is expressed as the number of haemagglutination units per mg protein.

2.6 Complement activation assay

Serum and aqueous extract were mixed in 1:1 dilution and was incubated with an equal volume of 2% erythrocyte suspension in PBS. The absorbance of haemoglobin released from RBC lysis was measured at 560 nm. Appropriate controls were kept for the analysis and ethanol was used to lyse the cells.

2.7 MTT Assay

In this study, K562 (Chronic myelogenous leukemia) cells cultured in RPMI-1640 media were used. The cells were maintained in 25cm^2 cell culture flask. K562 cells were sub cultured in the ratio 1:2 to 1:4 every 2-3 days, supplemented with 10% FBS in a humidified 5% CO_2 incubator. 150µl of medium containing 10^5 cells/ml were seeded in to each well of the microtitre plate except one well which serves as blank and the plates were incubated overnight. Different concentrations of the extracts were then added to the respective wells, one kept as control without adding the extract. Volume of each well was then made up to 200µl with respective media. Incubated the cells for 48 hours in CO_2 incubator at 37 °C, 5% CO_2. 20µl of the MTT solution (5mg/ml) was added to each well after removing the drug and the plates were incubated for further 3 hours in CO_2 incubator at 37 °C. At the end of incubation, spent medium was pipetted out and the formazan crystals were dissolved in 200 µl of DMSO. Readings were taken in UV spectrophotometer at 570 nm against a blank containing DMSO. The IC_{50} value was calculated using ED50plusV 1.0 software. The percentage of viability was calculated using the formula,

$$\text{Cell viability (\%)} = \frac{\text{Absorbance with plant extract}}{\text{Absorbance of control}} * 100$$

3. Results

3.1 Extraction of secondary metabolites

10g of *Tragia involucrata* leaf powder was subjected to single solvent extraction using different solvents such as petroleum ether (PE), chloroform (CHF), ethyl acetate (EA) and methanol (MOH) in the increasing order of their polarity.

The successful extraction of biologically active compounds from plant materials mainly depends on the type of the solvents used for the extraction procedure. In the case of single solvent extraction, methanol gave the best yield whereas petroleum ether gave the least yield (Figure 1).

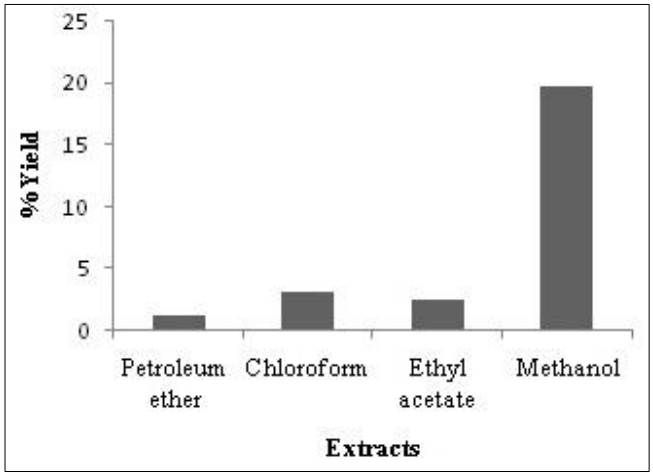


Fig 1: Percentage yield of *T. involucrata* extracts on single solvent extraction

3.2 Thin layer chromatographic identification of secondary metabolites

Thin layer chromatography is considered to be the first step to identify the phytochemical constituents present in the extract. It provides a chromatographic finger print of the secondary metabolites present in the respective solvent extract [10]. The extracts were spotted on the TLC plates and separated using suitable solvent system as mentioned in materials methods. The color of the spots under visible, short UV and long UV light and their R_f values were calculated and shown in Table 1 and the TLC chromatogram of the extracts are illustrated in Figure 2 for the solvent system petroleum ether: ethylacetate (6:4). Solvent combination Petroleum ether: Acetone (3:2) was also tried and the findings are represented in Table 2 and Figure 3. From this observation we could infer the presence of various phytoconstituents present in the extracts. No separation was observed in the methanol extract using this solvent system.

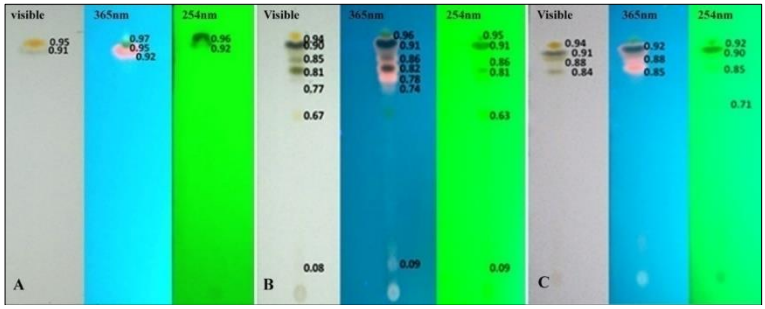


Fig 2: Thin layer chromatogram of A-petroleum ether, B- chloroform and C-ethyl acetate extracts of *T. involucrate* separated using petroleum ether: ethyl acetate (6:4) and observed under visible light, long UV 365nm and short UV 254nm

Table 1: Thin layer chromatographic identification of various solvent extract of *Tragia involucrata* using Petroleum ether: Ethyl acetate (6:4)

Extract	Visible		UV 365nm		UV 254nm	
	Color	R_f	Color	R_f	Color	R_f
Petroleum ether	Yellow	0.95	Blue	0.97	Black	0.96
	Green	0.91	Green	0.95	Green	0.92
			Red	0.92		
Chloroform	Bright yellow	0.94	Green	0.96	Green	0.95
	Dark green	0.90	Blue	0.91	Black	0.91
	Sage green	0.85	Red	0.86	Green	0.86
	Olive green	0.81	Red	0.82	Black	0.81
	Light green	0.77	Red	0.78	Green	0.63
	yellow	0.67	Green	0.74	Green	0.14
	Light yellow	0.08	White	0.09	Green	0.09
Ethyl acetate	Yellow	0.94	Blue	0.92	Green	0.97
	Dark green	0.91	Red	0.88	Black	0.92
	Light green	0.88	Red	0.85	Green	0.90
	Olive green	0.84			Green	0.85
					Green	0.71
Methanol	No characteristic separation					

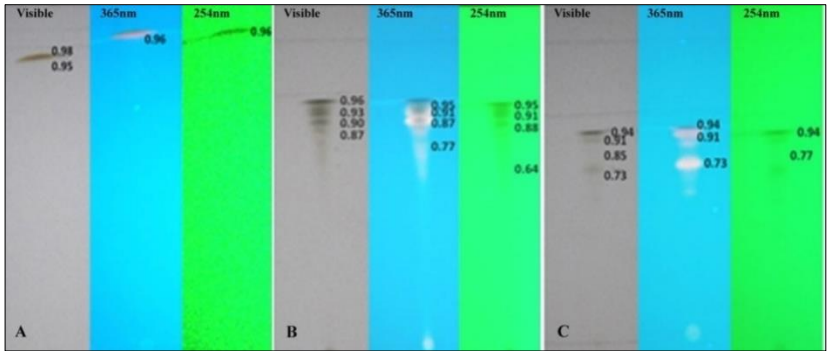


Fig 3: Thin layer chromatogram of A-petroleum ether extract, B- chloroform extract and C- ethyl acetate extract of *T. Involucrata* separated using petroleum ether: acetone (3:2) and observed under visible light, long UV 365nm and short UV 254nm

Table 2: Thin layer chromatographic separation of various solvent extract of *Tragia involucrata* in Petroleum ether: Acetone (3:2)

Extract	Visible		UV 365nm		UV 254nm	
	Color	R_f	Color	R_f	Color	R_f
Petroleum ether	Yellow	0.98	Red	0.96	Green	0.96
	Green	0.95			Green	0.95
					Green	0.88
					Green	0.75
Chloroform	Dark green	0.96	Blue	0.95	Green	0.95
	Light green	0.93	Red	0.91	Green	0.91
	Light green	0.90	Red	0.87	Green	0.88
	Light green	0.87	Red	0.77	Green	0.85
			Red	0.72	Green	0.80
					Green	0.64
Ethyl acetate	Dark green	0.89	Blue	0.94	Green	0.91
	Light green	0.85	Red	0.91	Green	0.87
	Light green	0.73	Red	0.73	Green	0.77
					Green	0.61
Methanol		No characteristic separation				

Of the solvent systems tested, the maximum separation of the secondary metabolites was obtained in the combination of Petroleum ether: ethyl acetate (6:4) and different spraying reagents were employed for their identification.

Anisaldehyde –Sulphuric acid test: Anisaldehyde-Sulphuric acid reagent was sprayed on the separated TLC plates. It detected the presence of sugars in PE extract; phenols, steroids, terpenoids and glycosides in CHF and steroids in EA. No reaction was found in MOH (Figure 4).

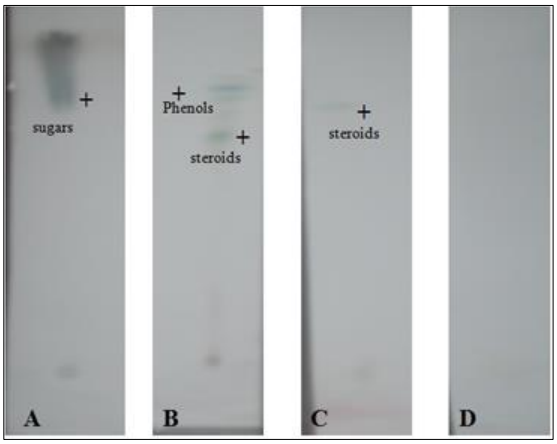


Fig 4: Anisaldehyde-Sulphuric acid test for the detection of phenols, sugars, steroids and terpenoids - (A) PE (B) CHF (C) EA and (D) MOH

Fast blue reagent test: Fast blue reagent was sprayed on the TLC plates after separation. The appearance of the pink spots indicated the presence of phenolic compounds. All the extracts except methanol showed the presence of phenolic constituents as shown in Figure 5.

Iodine test: TLC plates were placed in the chromatographic tank saturated with iodine vapours. The yellow coloration indicated the presence of compounds with conjugated double bonds. All the extracts showed the presence of compounds having conjugated double bonds (Figure 6).

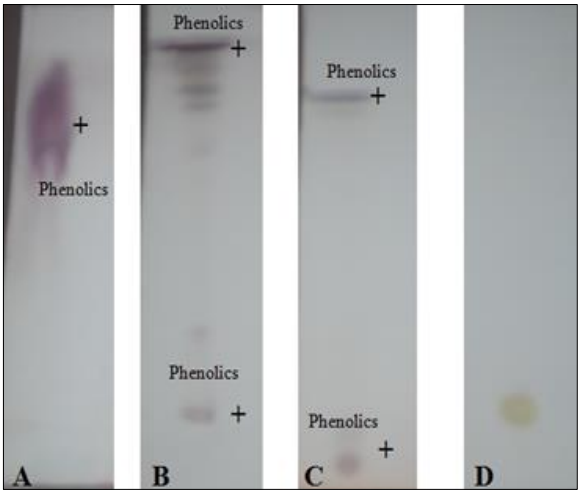


Fig 5: Fast blue reagent test for the detection of phenolic compounds. (A) PE (B) CHF (C) EA and (D) MOH

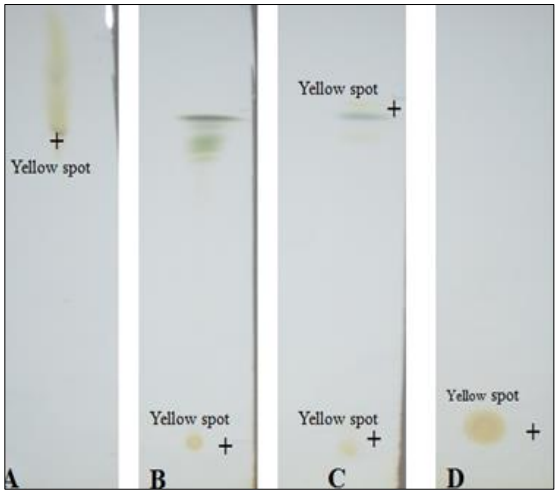


Fig 6: The iodine test for the detection of compounds having conjugated double bonds. (A) PE (B) CHF (C) EA and (D) MOH

Potassium hydroxide test: TLC plates were sprayed with potassium hydroxide and observed under visible light. Appearance of Red, yellow and blue spots after spraying indicates the presence of anthraquinones, anthrones and coumarins. Anthrone was detected in PE, EA and MOH and anthraquinone was detected only in EA as illustrated in figure 7.

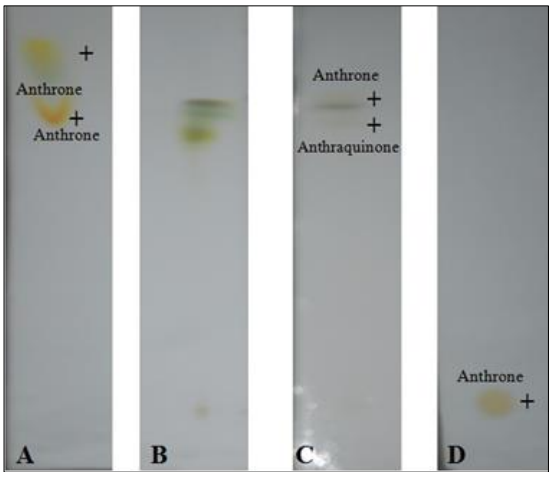


Fig 7: Potassium hydroxide test for the detection of anthraquinones, anthrone and coumarins. (A) PE (B) CHF (C) EA and (D) MOH

3.3 Antioxidant potential of *T. involucrata* using DPPH

After separation on TLC plates, the secondary metabolites with radical scavenging activity were determined with DPPH spraying reagent. The TLC plates were observed under visible light. As shown in Figure 8, the samples producing yellowish bands were considered as showing free radical scavenging ability. The background of the plate also changed from white to purple.

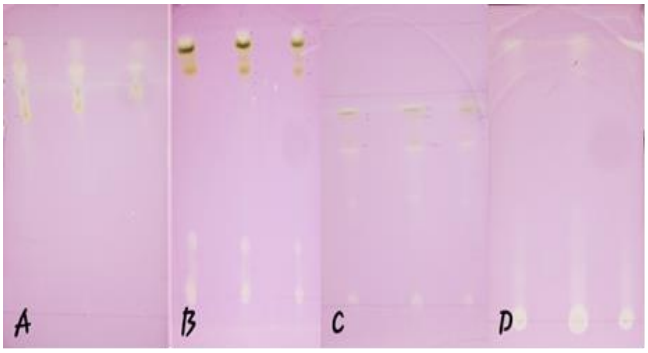


Fig 8: TLC plates fractionated in (A) Petroleum ether (chloroform: methanol[3:2]), (B) Chloroform (petroleum ether: ethyl acetate [6:4]),(C) Ethyl acetate (petroleum ether: acetone [3:2]), (D)methanol (petroleum ether: acetone [3:2]) extracts showing free radical scavenging activity.

3.4 Haemagglutination assay

Phytolectins are proteins or glycoproteins that are derived from plants and are having the ability to bind specifically to sugar moieties in cell walls and plasma membranes. The aqueous extract exhibited agglutination only on well against human erythrocytes with a titre value of 4 (Figure 9). 50 µl of sample were taken to determine the specific activity and was estimated as 200 HU/mg and the total protein in 1ml of extract was 0.4 mg. The total activity was found to be 80 HU.

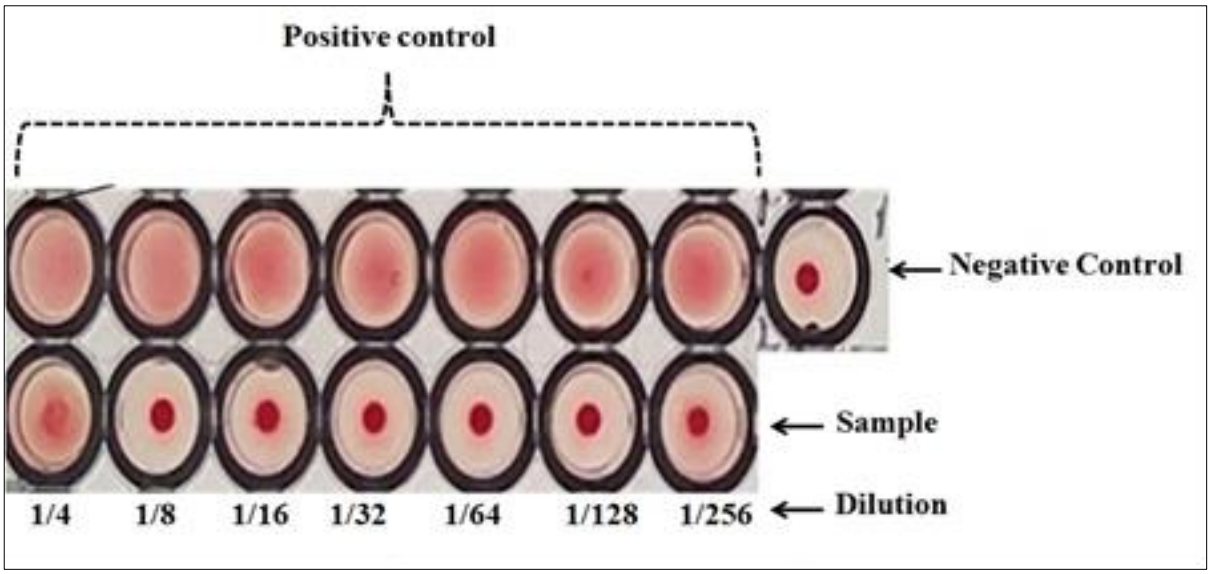


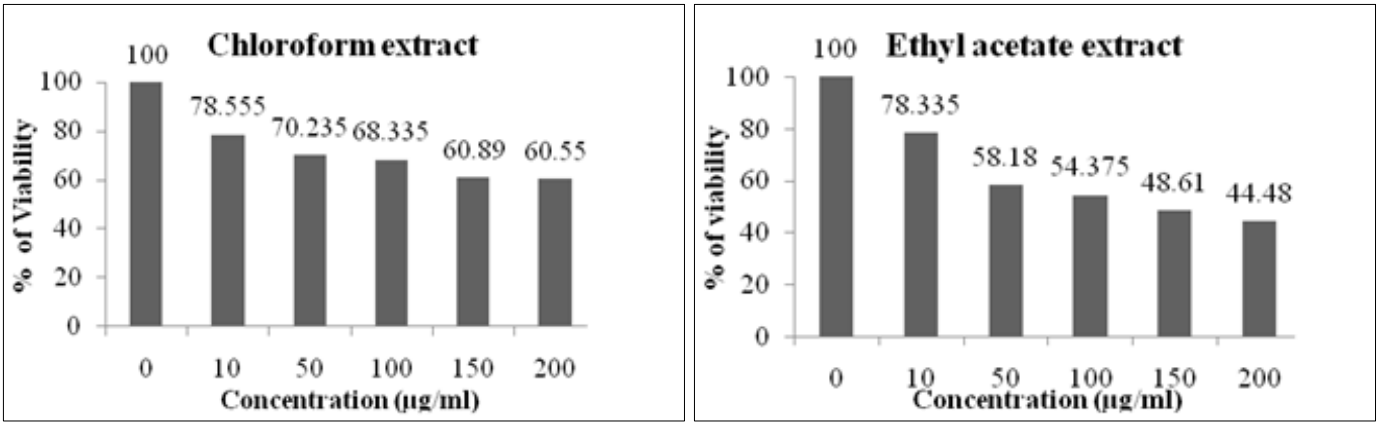
Fig 9: Aqueous extract showing haemagglutination potential of *T. Involucrata*

3.5 Complement activation test

Complement activation test was performed for the ability of plant extract to activate complement cascade in presence of RBC and there by lysing erythrocytes. Here there was no complement activating complement in the extract of *T. Involucrata*.

3.6 Antiproliferative activity using MTT Assay

The antiproliferative potential of *T. involucrata* was evaluated against K562 cell line. The cells were treated with different concentrations of the solvent extracts to identify their cytotoxic and antiproliferative action. The chloroform extract and ethyl acetate extract exhibited remarkable antiproliferative ability against K562 cell lines. The IC_{50} value of the chloroform extract was found to be 235 µg/ml and that of ethyl acetate extract was 181 µg/ml as calculated using ED50plus V1.0 software.



(a) (b)

Fig 10: Graph showing the antiproliferative property of *T. involucrata* on K562 cell line

4. Discussion

The present study investigated the presence of phytoconstituents in *Tragia involucrata* possessing cytotoxic, DPPH free radical scavenging, hemagglutinating, and complement fixing potential. The successful extraction of biologically active compounds from plant materials mainly depends on the type of the solvents used for the extraction procedure. In the case of single solvent extraction, methanol gave the best yield whereas petroleum ether gave the least yield. Preliminary phytochemical analysis with thin layer chromatography using different spraying reagents revealed the presence of steroids and phenolics in chloroform extract, anthraquinones in ethyl acetate extract. As it is evident from the TLC separation, using any one solvent for the extraction would have left some of the classes of phytocompounds unidentified. So it is essential to use different solvents varying in their polarities to extract and study phytocompounds.

The presence of secondary metabolites with free radical scavenging activity was determined using DPPH reagent. As shown in Figure 8, the samples producing yellowish bands were considered as free radical scavenging components of *Tragia involucrata*. The increased production of free radicals leads to many disease conditions like cardiovascular disorders, rheumatoid arthritis, diabetes, cancer etc. So the phytoconstituents exhibiting the free radical scavenging property can provide potential role in prevention of diseases [11]. It is already reported that the phenolic compounds possess antioxidant ability [12]. So the phenolic compounds present in the extracts of *T. involucrata* would possibly contribute to its free radical scavenging ability. Haemagglutination potential of the plant extract was determined against human RBCs. The plant extract showed only less activity against RBCs. Complement activation test was done to determine whether there was any component in the extract which can activate the complement system. There were no lysis of the RBCs observed visually and colorimetrically indicating the absence of complement activating component in the plant extract. Bradford protein assay were performed to determine the specific activity, total protein and total activity of the plant extract which showed only mild activity.

The antiproliferative potential of *T. involucrata* was evaluated against K562 cell lines. The cells were treated with different concentrations of the solvent extracts to identify their cytotoxic and antiproliferative action. The chloroform extract and ethyl acetate extract exhibited remarkable antiproliferative ability against K562 cell lines. The IC_{50} value of chloroform extract was found to be 235 µg/ml and that of ethyl acetate extract was 181 µg/ml calculated using ED50plus V 1.0 software. The ethyl acetate extract was found to possess the highest antiproliferative property among the extracts tested. The study has demonstrated the presence of steroids, phenolics, anthrones, anthraquinones and compounds with conjugated double bonds in the ethyl acetate extract. The chloroform extract had phenolics, steroids and compounds with conjugated double bonds in them. These compounds may be responsible for the antiproliferative activities. According to Joshi *et al*., 2001 the methanol and hexane extracts of *T. involucrata* showed cytotoxic activity on KB and MCF-7 cell lines, the presence of flavonoids in methanol and terpenoids in hexane fractions were ascertained [7]. The presence of the various phytochemicals present in the extract like phenols, terpenoids and steroids are connected and reported to reduce the risk of cancer [13, 14, 15].

5. Conclusion

Tragia involucrata is a plant in which very limited studies have been carried out. The present study revealed its phytochemical and cytotoxic properties. The clinical efficacy and extent of toxicity of numerous anticancer agents are unknown and uncertain. For example, research on majority of ayurvedic drugs is in the pre-clinical phase or is not being actively pursued. Future research on this topic would help to isolate the bioactives and identify the mechanism of antiproliferative activity exhibited by the extracts of *T. involucrata.*

6. Acknowledgements

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