Applied Biological Research 25(4): 424-436; (2023)
DOI: 10.5958/0974-4517.2023.00048.4

BIOLOGICAL POTENTIAL OF SEQUENTIALLY EXTRACTED LEAF EXTRACTS OF *Wrightia tinctoria* (Roxb.) R. Br.

V.S Soumya[1], Johnson Baby[2], K.P. Laladhas[1] and Sreejith Parameswara Panicker[3*]

[1]*St. Stephen's College, Pathanapuram, Affiliated to University of Kerala, Kollam – 689 695, Kerala (India)*
[2]*Christian College Chengannur, Affiliated to University of Kerala, Chengannur – 689 122, Kerala (India)*
[3]*Department of Zoology, University of Kerala, Karyavattom, Thiruvananthapuram - 695 581, Kerala (India)*
*e-mail: *p.sreejith@gmail.com*

(Received 20 June, 2023; accepted 12 September, 2023)

ABSTRACT

Wrightia tinctoria **has long been exploited for its therapeutic potential and has proved an effective natural medicine for various disorders and illnesses. This study was aimed to assess the phytochemical and biological activity of *W. tinctoria* extracts that would support both the traditional and pharmaceutical uses of its leaves. Following the consecutive extractions of fresh *W. tinctoria* leaves using petroleum ether, chloroform, methanol, and aqueous solvents in sequence; several phytochemicals were discovered. The study revealed the presence of alkaloids, flavonoids, tannins, beta-cyanin, fats and fixed oil in the extracts. The antioxidant activity of extracts using DPPH (2,2-diphenylpicryl hydrazyl) assay, antibacterial activity (disc diffusion assay), and the extract's apoptotic character (acridine orange and ethidium bromide staining) were also assessed. The study revealed relatively high bioactivity in chloroform extract. The chloroform extract showed good antioxidant (IC_{50} 296 µg mL^{-1}), anti-bacterial and anticancer (100 µg mL^{-1}) activity in SKBr3 cell lines. It was purified using column chromatography. The prominent fraction was chosen and characterized by GCMS analysis. Many active biomolecules with significant biological activity were found. Further studies are needed to understand the mechanism of action of these potent compounds noticed in *W. tinctoria* extract.**

Keywords: Bioactivity, characterisation, purification, *Wrightia tinctoria*

INTRODUCTION

The search for potential new therapeutics from traditional natural resources has been one of the priorities in pharmaceutical sector (Mateo *et al.,* 2001). Chemical prospecting is a joint endeavour of environmental activists, researchers and pharmaceutical corporates to develop products from the existing biodiversity and generate revenue to support its conservation for future generations (Reid *et al*., 1993). The exploitation of medicinal plants in recent times has grown significantly for the characterization, identification, and isolation of new natural compounds having therapeutic characteristics (Da *et al.*, 2007). Medicinal plants abundantly possess secondary metabolites including flavonoids, flavanones, anthocyanins, lignans, coumarins, isocatechins, and catechins. These bio-active compounds are liable for the biological property of medicinal plants (Gahtori *et al.,* 2023).

Wrightia tinctoria (Roxb.) R. Br. is used in the traditional Indian system of medicine for treating psoriasis and other skin-related problems. The plant appears as a life-saving treasure for many patients. *W. tinctoria* is a tiny deciduous tree of Apocynaceae family found in tropical India with scaly, smooth, ivory-coloured bark and milky latex. *Pala indigo* is the name given to the tree as it produces a blue dye in its leaves (Srivastava *et al.,* 2014). The preliminary phytochemical analysis of

W. tinctoria has shown the presence of flavonoids, alkaloids, phenols, saponins, and tannins (Vedhanarayanan *et al*., 2013). The plant reportedly contains sterols, desmosterol, clerosterol, 24-methylene-2-methyl cholesterol, 24-dehydro pollinastanal, terpene wrightal, and other phytoconstituents like cycloartenane and cycloleucalenol as well as other bioactive compounds like indigotin, isatin, indirubin, tryptanthrin, rutin (Amutha *et al.,* 2023). The other triterpenes found in plant's latex include β-sitosterol and β-amyrin, wrigatiadione, and wrightin, a stable serine protease (Jain *et al.*, 2010). Traditional healers employ the entire plant for various diseases as it contains hexadecanoic acid's critical bioactive component (Ramalakshmi *et al.,* 2012).

Yadala *et al.* (2020) reported a noticeable increase in the extract of *W. tinctoria* callus's capacity to scavenge the DPPH free radicals. Because of the structural needs and capacity for hydrogen donation, the flavonoids present in *W. tinctoria* are regarded essential for strong scavenging ability (Srivastava *et al.*, 2014). The highest antioxidant activity has been found in the leaves of *W. tinctoria*, which possess highest polyphenols and alkaloids contents, and have the ability to scavenge free radicals, which are involved in the pathophysiology of several diseases, including aging (Sawale *et al.*, 2014). Similarly, the tryptanthrin, a kind of indoloquinazolin present in *W. tinctoria* has antibacterial properties against various pathogens, including *Mycobacterium tuberculosis* (Frederich *et al.*, 2008). The bark and leaf extracts of *W. tinctoria* when exposed to sunlight reportedly have high antimicrobial activity against nine pathogenic bacteria, including *Salmonella paratyphi, Staphylococcus aureus, Escherichia coli, Proteus vulgaris, Klebsiella pneumoniae, Bacillus cereus, Enterobacter faecalis* and *Pseudomonas aerginosa*.

The aerial component extracts of *W. tinctoria* exhibit strong anticancer, immuno-modulatory, and anti-infective activity due to its phytoconstituents and their synergistic interactions (Devi *et al.*, 2019). The antiproliferative effects of *W. tinctoria* are linked with the induction of apoptosis in cervical and breast cancer cells (Fathima *et al.*, 2016). The anticancer triterpenoids lupeol and beta-sitosterol make the extract/fractions in breast cancer cells pro-apoptotic. Moreover, as they are more harmful to cancer cells than to healthy fibroblast cells, the fractions exhibit a preference for cancer cells (Chaudhary *et al*., 2015). It is a powerful bioactive substance that inhibits skin carcinogenesis by reducing inflammation and signaling pathway linked to β-catenin (Shankar *et al.,* 2020). Certain phytochemicals derived from *W. tinctoria* may be quantifiable, enabling their use as effective medications and learn more about the chemical composition of compound. Inspiration from an extensive spectrum of actions and biological activities of *W. tinctoria* extracts prompted us to look deeper into its constituents, and we are of the opinion that it may serve as an inexpensive candidate with least side effects. Attempts have been made in the present study to explore the presence of bioactive compounds in *Wrightia tinctoria* with the hope of finding some individual components which may prove a novel drug candidate for antibiotic, antioxidant and anticancer activity.

MATERIALS AND METHODS

Collection of plant material

The medicinal plant *Wrightia tinctoria* was collected locally from Thiruvananthapuram, Kerala (India) in December, 2022. Dr. S. Ghanthikumar, Research Officer (Botany) SRRI, Ayurveda College, Thiruvananthapuram, Kerala, India, authenticated the identity of plant specimen and a voucher specimen (06122104) was deposited at the institute.

Crude extract preparation and phytochemical analysis

The fresh leaves of *W. tinctoria* were rinsed under running water to remove the surface dust, then dried by air drying and ground in a mechanical grinder. The plant powders were extracted using a sequential extraction process using a variety of organic solvents, ranging from low polar to polar,

which included petroleum ether, chloroform, methanol, and ultimately aqueous. A separate airtight bottle containing 50 g of finely ground leaves was soaked in 500 mL petroleum ether and allowed to stand at room temperature for 1-2 days. Then, the material filtered using Whatman No. 1 filter paper after first passing through two layers of muslin fabric. Separate conical flasks were used to collect each filtrate. This process was carried out twice. The petroleum ether was eliminated from each filtrate using a rotary evaporator operating at a low temperature and reduced pressure, and the dried extracts were kept in the refrigerator until needed. Similar to the process used for petroleum ether extract, the residue was dried and then utilised for chloroform extraction, followed by methanol and water. The petroleum ether, chloroform, methanol, and aqueous extract of *W. tinctoria* were taken at 1 mg mL^{-1}, dissolved in appropriate solvents, and standard defined methodologies were employed for qualitative phytochemical screening and identification of different active components (Harborne, 1998).

Quantitative estimation of flavanoids

For estimation of flavonoids, the sequentially extracted *W. tinctoria* (1 mg mL^{-1}) extracts and standard rutin (1 mg mL^{-1}) were used. Then 100 µL potassium acetate (1 M), distilled water (2.8 mL), and 100 µL aluminum chloride (10% w/v) were mixed and allowed to stand for 30 min at room temperature. The absorbance of reaction mixture was measured at 415 nm using a UV-visible spectrophotometer ((Implen NanoPhotometer). The assay was compared to rutin equivalent using rutin as a standard material (Chang *et al*., 2002).

Quantitative estimation of alkaloids

To 1 mL test extract, 5 mL phosphate buffer (pH 4.7) and 5 mL bromocresol green solution were added, and the mixture agitated with 4 mL chloroform. The extracts were collected in 10 mL volumetric flask and diluted with chloroform to adjust the volume. The complex's chloroform absorbance was measured at 470 nm against a blank made similarly but without the extract. The assay was compared to atropine equivalent using atropine as a standard material (Madhu *et al*., 2016).

Screening for bioassays

The crude extract was subjected to various bioassays to understand its biocapability. The antioxidant, antimicrobial, and anticancer activity were checked to understand the bioactivity of the different crude extracts of *W. tinctoria*.

DPPH radicle scavenging assay: The DPPH (2,2-diphenyl-1-picyhydrazyl) free radicle scavenging capability of *W. tinctoria* crude leaf extracts in petroleum ether, chloroform, methanol, and aqueous was determined using the technique with minor changes. The 3.9 mg DPPH was dissolved in 50 mL methanol to make DPPH reagent solution (0.2 mM). Methanol was used to react to different concentrations of extract (100, 200, 300, and 400 g mL^{-1}) with 1 mL DPPH solution in a final volume of 3 mL. The mixture was vigorously stirred and kept as such at room temperature for 30 min. The decrease in the absorbance of resultant solution was measured spectrophotometrically (Implen NanoPhotometer) at 517 nm. The lower absorbance indicates a higher free radical scavenging capacity. The ascorbic acid (2, 4, 6, 8, 10, and 12 g mL) was used as standard, and the percentage of inhibition was represented on X-axis. The tests were repeated three times in total. The formula was used to calculate the IC_{50} values for the standard and samples (Sreeja *et al*., 2021).

$$\% \text{ Inhibition } = \frac{(\text{Abs of Control} - \text{Abs of Test})}{(\text{Abs of Control})} \times 100$$

Antimicrobial assay: The American Type Culture Collections of *Escherichia coli* (ATCC 25922)*, Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 23235), and *Enteroccocus feacalis* (ATCC29212) were procured from Pathogen Biology Laboratory, RGCB, Trivandrum, Kerala, India. These bacterial test strains were grown in nutritional broth. The disc diffusion method on Muller Hinton agar plates was used to assess the antibacterial susceptibility of *W. tinctoria* extracts against bacterial isolates *in vitro* (Hi-media) (Jain *et al.,* 2016). A single layer of Muller-Hinton agar

was placed into 1/3 of Petri dishes (100 mm) to a thickness of 2 mm and allowed to harden for 15 min. Following the solidification, 0.1 mL of 18-h bacterial shake culture was surface swabbed with sterile cotton swabs. Hi-media sterile discs (6 mm dia) were impregnated with 20 µL aliquots of *W. tinctoria* plant extract concentration (1mg mL) and placed on the inoculated agar surface using sterilized forceps in triplicate sets with adequate controls. The area of inhibition zone (mm) was measured using a Hi antibiotic zone scale C after 24 h incubation at 20 or 30°C in a BOD incubator (Tanco). The antibacterial potential of *W. tinctoria* extracts was compared with standard antibiotics (Hi-Media) such as ampicillin (10 mcg $disc^{-1}$). The zone of inhibition (including the well diameter) that emerged after the incubation period was measured to detect antimicrobial activity (Pramitha *et al.*, 2014).

Anticancer activity-AO/EB-dual staining: The SK-BR-3 (breast cancer) cell line procured from NCCS, Pune (India) were maintained in a culture medium of low glucose DMEM (Dulbecco's Modified Eagle Medium), supplemented with 5% (v/v) fetal bovine serum, 1% (v/v) antibiotic (100 U mL^{-1} Penicillin and 0.1 mg mL^{-1} streptomycin), at 37^0C, 5% CO_2 incubator. After reaching confluency, 2-5 x 10^6 cells were seeded in a 12-well culture plate and incubated for 24 h until it attained 90% confluence. The cells were treated with 100 µg mL^{-1} test drug. Media and trypsinized cells were centrifuged at 2000 rpm for 5 min. The pellets were resuspended in 50 µL PBS, stained with 1 µL AO/EB stain (1:1), and immediately observed under Leica fluorescent microscope using a 40X objective and a fluorescein filter (Ribble *et al.,* 2005).

Purification of crude extract of W. tinctoria

Thin-layer chromatography (TLC): Thin-layer chromatography analysis of *W. tinctoria* solvent extracts (petroleum ether, chloroform, methanol, aqueous) was used to detect the active compounds present. After activation at 110^0C for 30 min, TLC plates were run with 85% acetone and 15% petroleum ether and separated around 5µl of 0.1 mg mL^{-1} of different extracts of *W. tinctoria*. Under ultraviolet (UV) light, the separated components were observed. The migratory behaviour of isolated compounds was determined and reported as a retardation factor for the plates' qualitative evaluation (Rf value) (Hemavathy *et al*., 2019).

Column chromatography: A silica column of 60 cm x 3 cm size was used to fractionate 1 mL thick viscous chloroform extracts of *W. tinctoria*. Eluent ratios of n-hexane and acetone were utilized as the solvent system (n-hexane/acetone (%), 100:0, 80:20, 60:40, 40:60, 20:80, and 0:100). Because of their polarity and molecular nature, the plant extract compounds run down the column, forming bands. The fractions of the eluted amounts were collected progressively based on their colour (Jose *et al.,* 2014).

Spectroscopic analyses of GC-MS: GC-MS analysis of 1st fraction of chloroform extract of *W. tinctoria* was done on a combined GC-MS instrument (Shimadzu GC-MS Model: QP2010S) employing the following conditions: column ELITE-5MS of 30-m length, 0.25 mm depth, and 0.25 µm thickness. The samples (1 µL) were injected separately into the column using splitless injection at 280ºC. The GC column temperature was 60ºC for 2 min, increased to 260ºC for 10 min, and 280ºC for 6 min. The carrier gas was helium (1.5 mL min^{-1}). The mass source was set at 200ºC, and mass spectrometer adjusted to E1 mode. The peaks of chromatograms and spectra were closely monitored. The compounds present in samples were identified by comparing the mass spectral fragmentation patterns of each individual peaks in the chromatogram with GCMS software: GCMS Solutions and the Libraries NIST 11 & WILEY 8 (Sermakkani *et al.*, 2012).

Statistical analysis

All the experiments or analysis were carried out in triplicate. The data was analysed using one-way ANNOVA and the results presented as mean ± standard deviation. Significant differences between the groups were performed using SPSS program (SPSS Inc. Chicago, IL, USA). A p-value less than 0.01 was considered statistically significant.

RESULTS AND DISCUSSION

Phytochemical analysis of Wrightia tinctoria

Phytochemical screening or qualitative analysis is used to reveal the chemical constituents or the secondary metabolites of the plant extract. In present study, the preliminary phytochemical screening revealed the presence of alkaloids, flavonoids, tannin, fat and fixed oil (Table 1) in different extracts of *W. tinctoria*. Alkaloids were present in petroleum ether, chloroform and methanol extracts but absent in aqueous extract. Flavonoids were present in petroleum ether, chloroform and methanol extracts but absent in aqueous extract. Fats and fixed oil were present in chloroform, methanol and aqueous extracts but absent in petroleum ether extracts. Tannins were present in petroleum ether and chloroform extract but absent in methanol and aqueous extract. The positive tests were noted as low (+), moderate (++), high (+++), and absent (-). The results indicated the presence of active phytocompounds in chloroform extract > methanol extract > petroleum ether extract > aqueous extract. The phytochemical screening revealed the presence of flavonoid and alkaloids and these compounds can be utilized to prevent and treat free radical damage. Several earlier reports have also confirmed that free radicals play a significant role in an individual's disease scenario. These substances may also have antioxidant and anti-inflammatory properties (AN *et al.,* 2016).

Table 1: Preliminary phytochemical analysis of a different extract of *Wrightia tinctoria*

S. No.	Phytochemicals	Petroleum ether extract	Chloroform extract	Methanol Extract	Aqueous extract
1.	Alkaloid (Dragendroff test)	++	+++	++	+
2a.	Flavanoid (alkaline NaOH test)	++	+++	++	+
2b.	Flavanoid (ammonium test)	++	+++	+	+
3.	Terpenoid (Salkowski test)	++	++	-	-
4.	Quinone (sulphuric acid test)	+	+	++	++
5.	Phenol (ferric chloride test)	_	_	_	_
6.	Saponin (foam test)	+	+	++	+
7.	Fats and fixed oil	+	+++	+++	+++
8.	Anthraquinone	-	+	-	-
9.	Coumarins (fluorescence test)	+++	+	++	++
10.	Anthocyanin	+	-	-	-
11.	Betacyannin	+++	++	+++	+
12.	Tannin	++	+++	+	++
13.	Reducing sugar	-	+++	-	-
14.	Cardiac glycosides (Keller-Killani test)	+	+	_	_

* +++ = High concentration, ++ = Moderate concentration, + = Low concentration, - = Absent

Quantitative estimation of flavanoids

Flavonoids play vital role in scavenging the free radicals to prevent or reduce the oxidative damage encountered in human degenerative diseases (Pham-Huy *et al.,* 2008). In present study rutin was taken

Table 2: Total flavonoid content (μg mL^{-1}) in different extracts of *Wrightia tinctoria*

Different extracts of *W. tinctoria*	Flavanoid (μg mL^{-1} extract)	Alkaloid (μg mL^{-1} extract)
Petroleum ether extract	18.33±0.01	18.02±0.005
Chloroform extract	213.17±0.04	196.31±0.015
Methanol extract	23.81±0.02	32.33±0.005
Aqueous extract	13.47±0.005	11.02±0.05

The table shows the ug of flavonoid and alkaloid obtained per mg mL^{-1} of different extracts of *W. tinctoria*. The result obtained are expressed as mean ± SD (n = 3) with significant differences at $p < 0.01$ by Duncan's test with CD value flavonoid -24.80 and alkaloid -108.91.

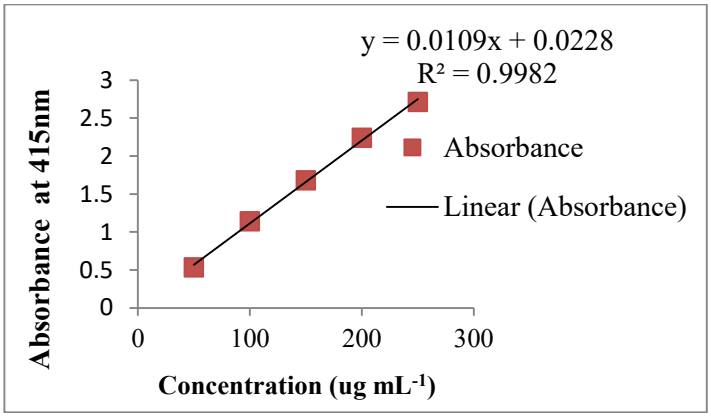

Fig. 1: Standard calibration curve for rutin (flavanoid)

as standard flavonoid. All the extracts of *W. tinctoria* contained flavonoids as essential phyto-constituents. Chloroform extract of *W. tinctoria* (1 mg mL^{-1}) showed higher flavonoid content, of 213.5 µg; however, in methanol extract it was 23.9 µg, petroleum ether extract 18.33 µg and aqueous found in leaves of *W. tinctoria* may be responsible for the above functions (Khyade *et al.,* 2014). extract 13.47 µg. The flavonoid content in different extracts of *W. tinctoria* was determined using the equation y = 0.0109X + 0.0228, (where X is rutin equivalent, and Y is absorbance (Fig. 1 & 2; Table 2). It seems probable that the alkaloid & flavonoid compounds indigotin, indirubin, trypanthin, isatin, and rutin noticed in the leaves of *W. tinctoria* may be responsible for the aforementioned activities (Khyade *et al.,* 2014).

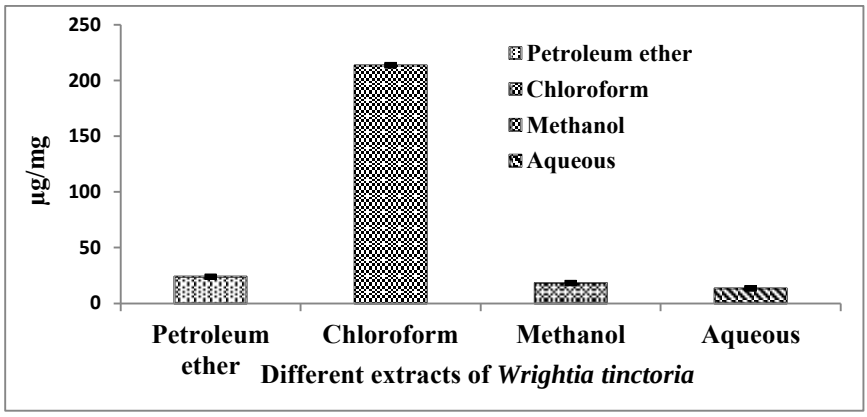

Fig. 2: Total flavonoid content in different extracts of *Wrightia tinctoria*

Quantitative estimation of alkaloid

Alkaloids have been used for medicinal and therapeutic purposes. This beneficial metabolite is expected to help in the treatment of many fatal diseases like cancer (Kaur *et al.,* 2015). To estimate the total alkaloid, atropine was used as standard, and a calibration curve was plotted in different concentrations (µg mL^{-1}). According to the calibration curve, the alkaloid content in the different extracts of *W. tinctoria* was determined using the equation Y = 0.0026X - 0.0138, where X is atropine equivalent, and Y is absorbance (Fig. 3). Total alkaloid of *W. tinctoria* was estimated and data is presented in Table 2 and Fig. 4. Chloroform extract of *W. tinctoria* showed increasing alkaloid content, which was 196 µg in chloroform extract, and others were 32 µg in methanol extract and petroleum ether extract 18 µg and aqueous extract 11 µg. The test leaf extracts appeared a good source for further pharmacological research due to the presence of high concentration of active phytoconstituents like phenols, flavonoids, alkaloids, terpenes, etc. As per Vedhanarayanan *et al.* (2013), the ethanolic leaf extract of *W. tinctoria* has flavonoids, phenols, steroids, and tannins, while the leaf extracts in chloroform and methanol have alkaloids, saponins, steroids, and tannins.

TLC analysis of different extracts of W. tinctoria

Thin-layer chromatography analysis was used to assess the presence of phytochemical elements in different *W. tinctoria* solvent extracts (petroleum ether, chloroform, methanol, and aqueous). The separated bands were visualized under ultraviolet (UV) light, and the separated components' respective Rf value was measured. The results indicated the presence of active phytocompounds in all

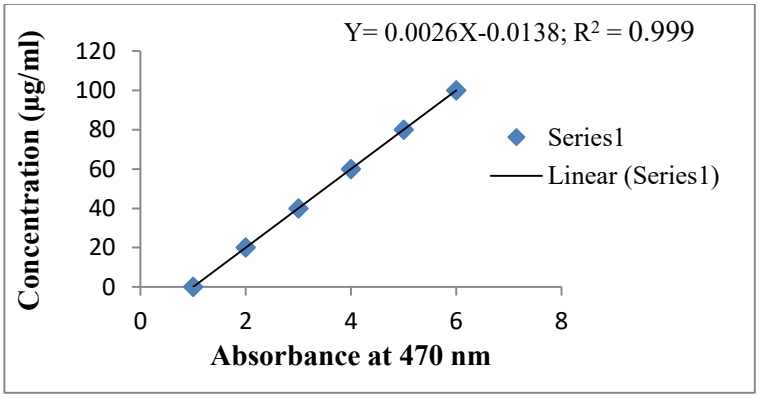

Fig. 3: Standard calibration curve for atropine (alkaloid)

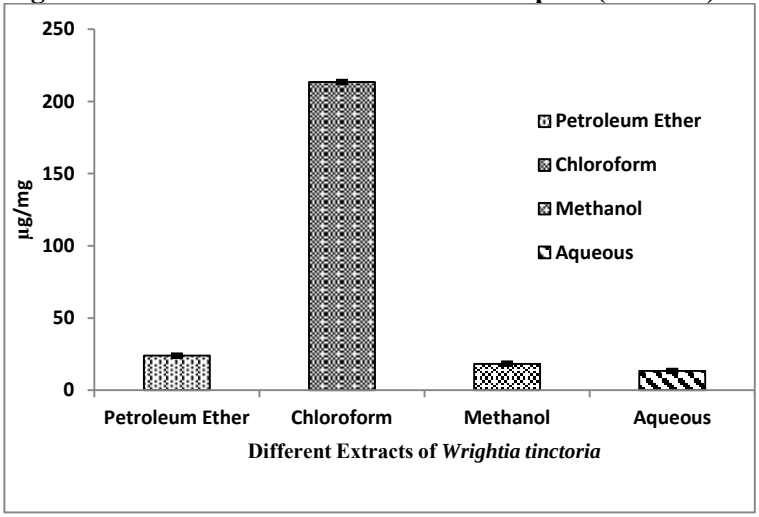

Fig 4: The alkaloid content in *Wrightia tinctoria* extracts

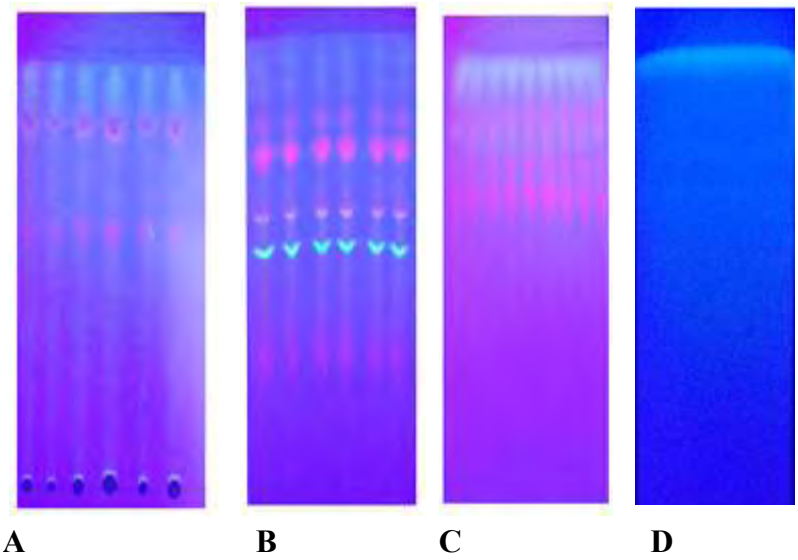

Fig. 5: Thin layer chromatography images of *Wrightia tinctoria* extracts A) Petroleum ether extract, B) Chloroform extract, C) Methanol extract, and D) Aqueous extract

the four extracts with their respective bands (Table 3; Fig. 5). Five bands were noted in chloroform, four in methanol, four in petroleum ether, and one in aqueous extract. The presence of visible bands in the methanol extract of *W. tinctoria* were noted by (Hemavathy *et al.,* 2019).

DPPH radical scavenging assay of W. tinctoria extracts

The antioxidant activity of petroleum ether, chloroform, methanol, and aqueous extract of *W. tinctoria* was estimated using DPPH free radicle assay (2,2-diphenyl-1-picryl-hydrazyl-hydrate). A significant increase in DPPH-free r adicle scavenging was noticed in chloroform extract of *W. tinctoria*. The results were compared to the ascorbic acid standard (Fig. 6). The results showed that the antioxidant activity goes up in order as the concentration goes up (Fig. 7). The IC_{50} values or the inhibiting concentration of extract were calculated using the formula Y = 7.85X + 0.3, R^2 = 0.9958 of ascorbic acid, where X is the ascorbic acid, where X is the ascorbic acid equivalent, and Y is the absorbance. The higher free radicals which have a scavenging action were observed in the chloroform extract, followed by methanol, petroleum ether, and aqueous extract (Table 4 and Fig. 8). The study revealed the presence of phenolic chemicals, particularly the flavonoids, which have numerous anti-oxidant characteristics and are deemed

Table 3: TLC Peaks and corresponding Rf values of different extracts of *Wrightia tinctoria*

Solvent of extract	No. of peaks	Rf value
Petroleum ether	4	0.25, 0.30, 0.48, 0.73
Chloroform	5	0.23, 0.5, 0.58, 0.71, 0.88
Methanol	4	0.53, 0.63, 0.8, 0.92
Aqueous	1	1.0

necessary for the development of efficient, intense free radicle scavenging (Sawale *et al.,* 2014) and this may be the reason for the potent antioxidant activity of chloroform extract of *W. tinctoria.*

Table 4: DPPH assay (% inhibition) of different leaf extracts of *Wrightia tinctoria*

Concentration ($\mu g\ mL^{-1}$)	% of inhibition			
	Petroleum ether extract	Chloroform extract	Methanol extract	Aqueous extract
100	14.42 ± 0.005	18.15 ± 0.005	14.11 ± 0.010	12.13 ± 0.005
200	19.31 ± 0.005	42.20 ± 0.005	25.93 ± 0.050	16.06 ± 0.005
300	41.16 ± 0.000	52.71 ± 0.010	48.11 ± 0.010	39.17 ± 0.010
400	50.20 ± 0.010	62.21 ± 0.010	67.15 ± 0.005	45.21 ± 0.010
C_{50} ($\mu g\ mL^{-1}$)	398	296	312	577

The table shows the free radicle scavenging activity of different extracts of *Wrightia tinctoria*. The result obtained are expressed as mean ± SD (n = 3) with significant differences at $p < 0.01$ by Duncans test at a CD value of -11.85.

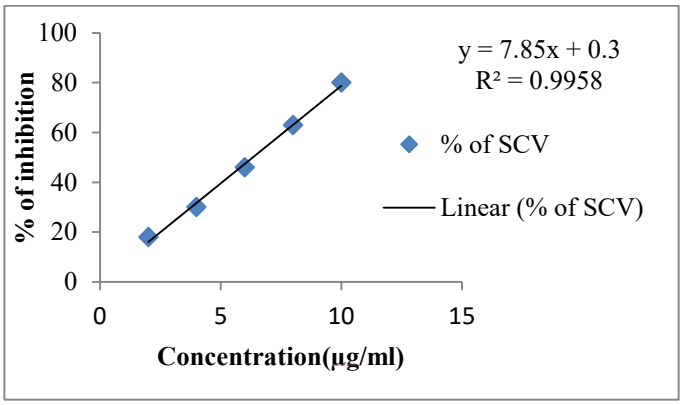

Fig. 6: DPPH assay of ascorbic acid as standard

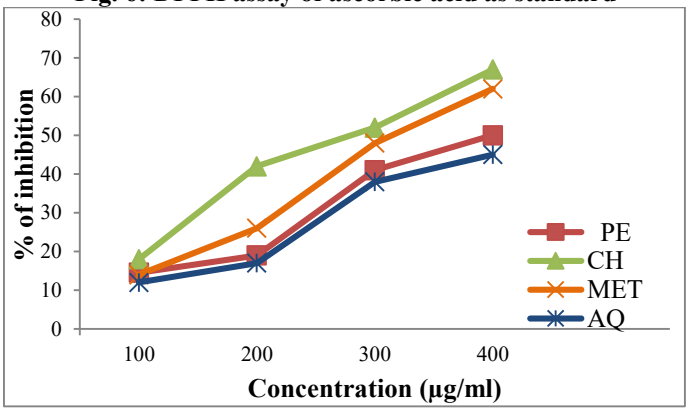

Fig. 7: DPPH assay of different extracts of *Wrightia tinctoria;* PE - Petroleum ether, CH - Chloroform, MET - Methanol, AQ - Aqueous extracts of *W. tinctoria*

Antimicrobial properties of different extracts of W. tinctoria

W. tinctoria leaf petroleum ether, chloroform, methanol, and aqueous extracts were screened against human non-pathogenic and pathogenic bacteria using disc diffusion method. Compared to the positive control, ampicillin, chloroform, and methanol extracts showed substantial antibacterial activity against human pathogenic bacteria. The chloroform extract showed highest antibacterial against *E. coli* (20 mm), *Pseudomonas aeruginosa* (16 mm), *Staphylococcus aureus* (16 mm), *Enterococcus faecalis* (6 mm). The methanol extract also showed such activity against *E. coli*, *Ps. aeruginosa*, and *E. faecalis*. Similarly, petroleum ether extract showed *E. coli*, *P. aeruginosa*, *S. aureus*, and *E. faecalis.* The aqueous extract of *W. tinctoria* leaf showed antibacterial activity against *E. coli*, *P. aeruginosa*, *S. aureus*, and *E.*

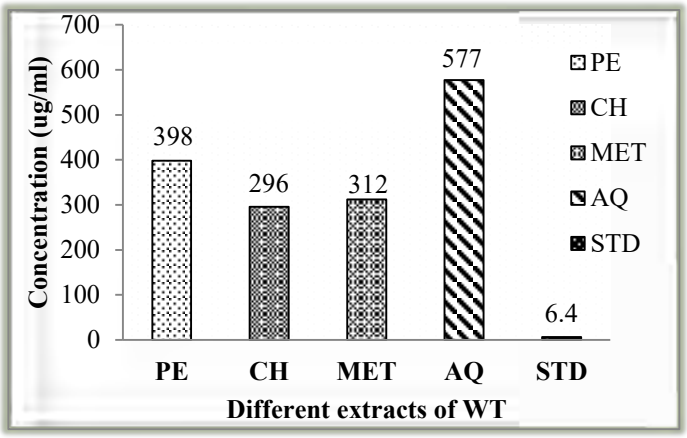

Fig. 8: IC $_{50}$ values of different extracts of *W. tinctoria;* PE- petroleum ether, CH - chloroform, MET - methanol, AQ - aqueous extracts of *W. tinctoria*; STD - ascorbic acid

faecalis. This suggests that the active ingredients can serve as potential drug candidates for treating bacterial diseases (Fig. 9; Table 5). The anti-bacterial property of crude hexane, chloroform, ethyl acetate, methanol and aqueous leaf extracts of *W. tinctoria* has been evaluated using agar well diffusion method and the extract showed potent antibaterial activity (Maddila *et al.,* 2017; Beena *etal.,* 2014). The antibacterial property seen in chloroform extract of *W. tinctoria* may be due to these active phytoconstituents, which may break the peptidoglycan layer in bacteria and cause membrane disruption.

Acridine orange/ethidium bromide staining of W. tinctoria extracts

To confirm the apoptotic and antiproliferative nature of different extracts of *W. tinctoria,* acridne orange/ethidium bromide assay was performed in Skbr3 cell lines with extracts treatment (100 µg mL^{-1}) for 24 h. All cells absorbed acridine orange dye, which turned nuclei luminous green, while ethidium bromide dye turned dead cells orange. Further, ethidium bromide replaced the acridine orange stain. As a result, live cells were green, early apoptotic cells were yellow, and late apoptotic cells were orange or red. Under fluorescence microscope, the chloroform extract of *W. tinctoria* was found to cause apoptosis in Skbr3 cell lines (Fig. 10). Green colors were homogeneous in control group, and the morphology was typical. Compared to other *W. tinctoria* extracts and the vehicle 0.1% DMSO, cells treated with chloroform extract had much higher apoptotic activity. Our results with the chloroform extract of plant confirm the apoptotic induction in breast cancer cell lines which further demonstrates its potential role as a chemotherapeutic agent. Earlier reports also indicate that *W. tinctoria's* phyto-chemicals and phenolic compounds can suppress cell growth and induce apoptosis in breast cancercells(Fathima*etal.,* 2016).

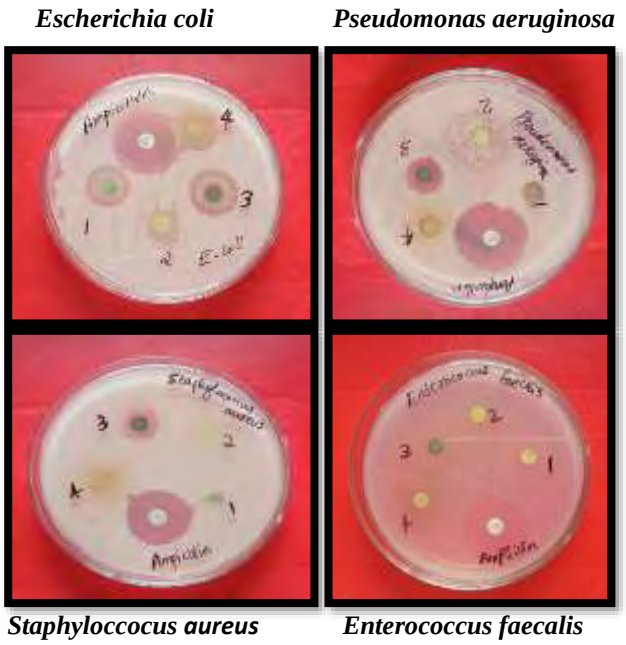

Fig. 9: Antimicrobial property of different extracts of *W. tinctoria*; 1) petroleum ether extract, 2) methanol extract, 3) chloroform extract, and 4) aqueous extract

Purification of chloroform extract of W. tinctoria through column chromatography

The sequentially extracted chloroform extract of *W. tinctoria* having bioactivity was further selected for purification through column chromatography. The *W. tinctoria* chloroform extract compounds started eluting at 80:20 ratio of petroleum ether and acetone at a flow rate of 2 mL min^{-1} (Table 6). The fraction was subjected to TLC, and active fraction was characterized through GC-MS analysis.

Table 5: Antimicrobial property (inhibition zone in mm) of different leaf extracts of *W. tinctoria*

Microorganisms	Positive control (ampicillin 10 µg g^{-1})	Petroleum ether extract	Chloroform extract	Methanol extract	Aqueous extract
Escherichia coli	25	15	20	15	16
Pseudomonas aeruginosa	29	10	16	20	10
Staphylococcus aureus	24	6	16	-	10
Enterococcus faecalis	30	6	6	6	6

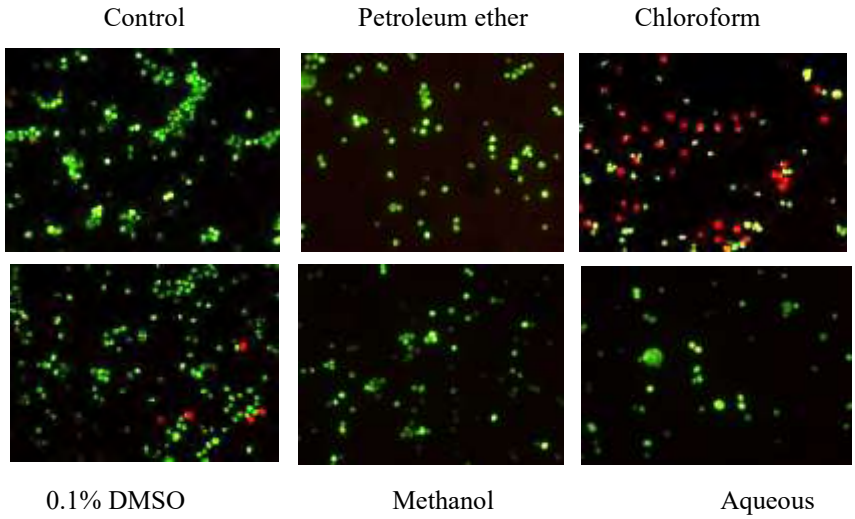

Fig. 10: Acridine orange /ethidium bromide staining of different extracts of *Wrightia tinctoria*

Table 6: The fractions isolated from the chloroform extract of *Wrightia tinctoria*

Petroleum ether: acetone	Chloroform fractions	Fraction colour
100:0	1	-
80:20	6	1 - Grey 2 - Blue 3 - Pink 4 - Purple 5 - Orange 6 – Yellow
60:40	2	7 - light green 8 - light yellow
40:60	1	-
20:80	1	-
0:100	1	-

GC-MS analysis of 1st fraction of chloroform extract of W. tinctoria

GC-MS analysis of 1st fraction of chloroform extract of *W. tinctoria* extract obtained from coloumn chromatography was carried out using the Shimadzu GC-MS model. The retention time and mass spectra of compounds in the fraction and the area's ratio of distinctive ions were matched with those of the relevant standards with the libraries of NIST 11 and Wiley. Ions with the mass /charge ratio(m/z) are shown in (Table 7 and Fig. 11). We identified 13 compounds, of which 3 major compounds were hexadecane 26%, isohexadecane 17%, nonadecane 11% with an m/z ratio of 57 and minor ones are benzene, Benzene,1,3-bis(1,1-dimethylethyl) 6%, tetradecane 5.6%, octadecane 3.8%, pentadecane 3.9%, 8-pentadecanone 2.7%, E-15-heptadecenal 6.8%, 8-octadecanone 2.3%, dotriacontane 4.6%, phytol 5.4%, 1-heneicosanol 4.7%. Some of these compounds are reported to be having antimicrobial and anticancer activity (Srivastava *et al.,* 2015). The presence of hexadecane, isohexadecane, nonadecane and other compounds in the respective fraction may be responsible for anticancer, antioxidant and antibacterial activities (Balamurugan *et al.,* 2013). It is reasonable to doubt that these compounds may have antimicrobial and anticancer activities. Our results are in conformity with the reports of several earlier investigators.

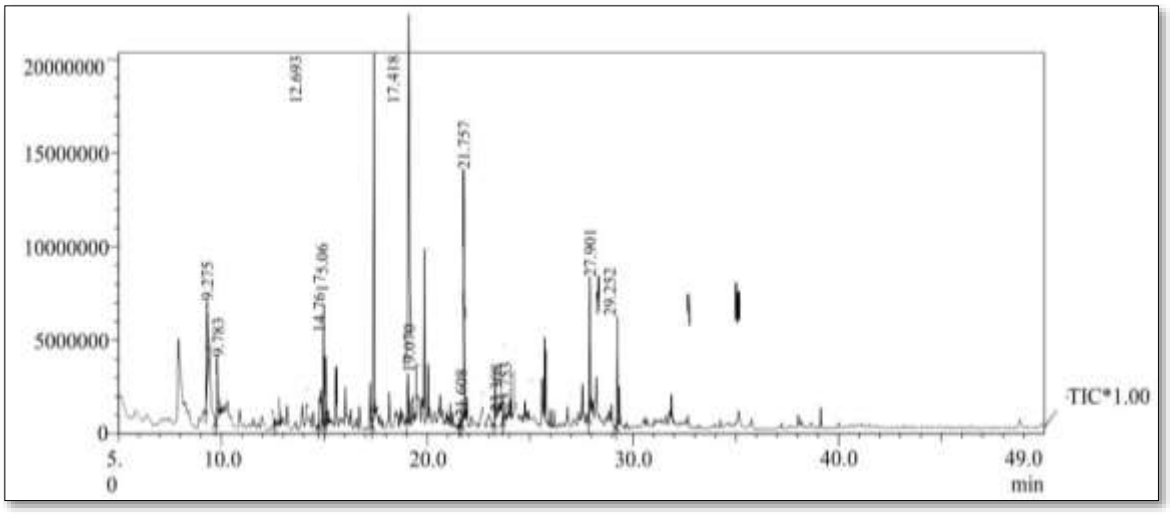

Fig. 11: The peaks and compounds identified in the 1st fraction of chloroform extract of *Wrighta tinctoria;* X-axis shows the area and Y-axis shows retention time

Table 7: The peaks and the compounds identified in 1st fraction of chloroform extract of *W. tinctoria*

Retention time (min)	Name of the compound	Peak area (%)	Molecular weight
9.275	Benzene,1,3-bis(1,1-dimethylethyl)	6.17	175.15
9.783	Tetradecane	5.61	57.10
12.693	Hexadecane	26.14	57.10
14.767	Octadecane	3.80	57.10
15.068	Pentadecane	3.86	57.05
17.418	Isohexadecane	17.25	57.05
19.070	8-Pentadecanone	2.72	57.05
21.608	e-15-Heptadecenal	6.76	55.05
21.757	Nonadecane	10.58	57.05
23.308	8-Octadecanone	2.32	57.05
23.753	Dotriacontane	4.65	57.05
27.901	Phytol	5.44	71.05
29.252	1-Heneicosanol	4.71	57.05

Conclusion: This study examined the biological and pharmacological aspects of several solvent extracts of *W. tinctoria* leaves. Even though all extracts had potential bioactivity, chloroform extract was found to have a high activity level when compared to other extracts. In the light of these observations, it is reasonable to assume that *W. tinctoria* phytocompounds have a potent pharmaceutical role and can be employed as a promising candidate for antibiotic, antioxidant and anticancer activity. However, additional research is required to thoroughly understand the mechanism of action of these active ingredients from this plant.

Acknowledgment: The author thanks the Council of Scientific and Industrials Research, New Delhi, India, for Senior Research Fellowship (08/666(0003)/2019-EMR-I and AcREM stem, University of Kerala, for the facilities to carry out this investigation.

REFERENCES

Amutha, E., Sivakavinesan, M., Rajaduraipandian, S. and Annadurai, G. 2023. Identification of phytochemicals capping the biosynthesized silver nanoparticles by *Wrightia tinctoria* and evaluation of their *in vitro* antioxidant, antibacterial, antilarvicidal, and catalytic activities. *Emergent Materials*, **6**(2): 525-534.

Balamurugan, M., Selvam, G.G., Thinakaran, T. and Sivakumar, K. 2013. Biochemical study and GC-MS analysis of *Hypnea musciformis (*Wulf*.) Lamouroux*. *American-Eurasian Journal of Scientific Research*, **8**(3): 117-123.

Chang, C.C., Yang, M.H., Wen, H.M. and Chern, J.C. 2002. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *Journal of Food and Drug Analysis*, **10**(3): 178-182.

Chaudhary, S., Devkar, R.A., Bhere, D., Setty, M.M. and Pai, K.S.R. 2015. Selective cytotoxicity and pro-apoptotic activity of stem bark of *Wrightia tinctoria* (Roxb.) R. Br. in cancerous cells. *Pharmacognosy Magazine*, **11**(Suppl 3), S481. [*doi: 10.4103/0973-1296.168976*].

da Graça Rocha, G., Simoes, M., Lúcio, K.A., Oliveira, R.R., Kaplan, M.A. and Gattass, C.R. 2007. Natural triterpenoids from *Cecropia lyratiloba* are cytotoxic to both sensitive and multidrug-resistant leukemia cell lines. *Bioorganic and Medicinal Chemistry*, **15**(23): 7355-7360.

Devi, N., Gupta, A.K. and Prajapati, S.K. 2019. Comparative **a**ntiproliferative activity of aerial parts of few Apocynaceae plants in HepG2, HT29 and SK-OV3 human cancer cell lines. *Journal of Drug Delivery and Therapeutics*, **9**(4): 1195-1202**.**

Fatima, N., Ahmad, M.K., Ansari, J.A., Ali, Z., Khan, A.R. and Mahdi, A.A. 2016. Anticancer, antioxidant potential and profiling of polyphenolic compounds of *Wrightia tinctoria* Roxb. (R. Br.) bark. *Journal of Advanced Pharmaceutical Technology and Research*, **7**(4): 159. [*doi: 10.4103/2231-4040.191428*].

Frederich, M., Tits, M. and Angenot, L. 2008. Potential antimalarial activity of indole alkaloids. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **102**(1): 11-19.

Gahtori, R., Tripathi, A.H., Kumari, A., Negi, N., Paliwal, A., Tripathi, P. and Upadhyay, S.K. 2023. Anticancer plant-derivatives: Deciphering their oncopreventive and therapeutic potential in molecular terms. *Future Journal of Pharmaceutical Sciences*, **9**(1): 14. [*https://doi.org/10.1186/s43094-023-00465-5*].

Harborne, A.J. 1998. *Phytochemical Methods a Guide to Modern Techniques of Plant Analysis*. Springer Science & Business Media. UK. [*https://doi.org/10.1007/978-94-009-5570-7*].

Hemavathy, A., Shanthi, P., Sowndharya, C., Thiripura, S.S. and Priyadharshni, K. 2019. Extraction and isolation of bioactive compounds from a therapeutic medicinal plant - *Wrightia tinctoria* (Roxb.) R. Br. *International Journal of Pharmacognosy and Phytochemical Research*, **11**(3): 199-204.

Jain, P.S. and Bari, S.B. 2010. Isolation of lupeol, stigmasterol and campesterol from petroleum ether extract of woody stem of *Wrightia tinctoria*. *Asian Journal of Plant Sciences*, **9**(3): 163-167.

Jain, S., Rathod, N., Nagi, R., Sur, J., Laheji, A., Gupta, N. and Prasad, S. 2016. Antibacterial effect of *Aloe vera* gel against oral pathogens: An *in vitro* study. *Journal of Clinical and Diagnostic Research* **10**(11), ZC41. [*doi: 10.7860/JCDR/2016/21450.8890*].

Jose, S.R. and Jesy, E.J. 2014. Evaluation of antibacterial and antifungal activities of the leaf and bark extracts of *Wrightia tinctoria* R.Br.. *World Journal of Pharmaceutical Research*. **3**: 4849-4859.

Kaur, R.A.J.B.I.R. and Arora, S.A.R.O.J. 2015. Alkaloids-important therapeutic secondary metabolites of plant origin. *Journal of Critical Reviews*, **2**(3): 1-8.

Khyade, M.S. and Vaikos, N.P. 2014. *Wrightia tinctoria* R. Br. - A review on its ethnobotany, pharmacognosy and pharmacological profile. *Journal of Coastal Life Medicine*, **2**(10): 826-840.

Madhu, M., Sailaja, V., Satyadev, T.N.V.S.S. and Satyanarayana, M.V. 2016. Quantitative phyto-chemical analysis of selected medicinal plant species by using various organic solvents. *Journal of Pharmacognosy and Phytochemistry*, **5**(2): 25-29.

Mateo, N., Nader, W. and Tamayo, G. 2001. Bioprospecting. *Encyclopedia of Biodiversity*, **1**: 471-487.

Pramitha, V.S. and Lipton, A.P. 2014. Antimicrobial effect of *Ulva fasciata* Delile, 1813 solvent extracts against multidrug resistant human pathogenic bacteria and fish pathogens. *Indian Journal of Geo-Marine Science*, **43**(12): 2244-2253.

Ramalakshmi, S., Edaydulla, N., Ramesh, P. and Muthuchelian, K. 2012. Investigation on cytotoxic, antioxidant, antimicrobial and volatile profile of *Wrightia tinctoria* (Roxb.) R. Br. flower used in Indian medicine. *Asian Pacific Journal of Tropical Disease*, **2**: S68-S75.

Reid, W.V., Laird, S.A., Gámez, R., Sittenfeld, A., Janzen, D.H., Gollin, M.A. and Juma, C. 1993. A new lease on life. pp. 1-52. **In**: *Biodiversity Prospecting: Using Genetic Resources for Sustainable Development.* (ed. S.A. Laird). World Resources Institute, USA/ Instituto Nacional de Biodiversidad, Costa Rica/ Rainforest Alliance, USA/ African Centre for Technology Studies (ACTS), Nairobi, Kenya.

Ribble, D., Goldstein, N.B., Norris, D.A. and Shellman, Y.G. 2005. A simple technique for quantifying apoptosis in 96-well plates. *BMC Biotechnology*, **5**(1): 1-7.

Sawale, J.A., Panchal, C.V., Padmane, S., Poul, B.N. and Patel, J.R. 2014. *In vitro* antioxidant and anti-inflammatory activity of *Wrightia tinctoria* leaves. *World Journal of Pharmaceutical Sciences*, **3**: 964-972.

Sermakkani, M., and Thangapandian, V. 2012. GC-MS analysis of *Cassia italica* leaf methanol extract. *Asian Journal of Pharmaceutical and Clinical Research*, **5**(2): 90-94.

Shankar G.M., Alex, V.V., Nisthul A.A., Bava, S.V., Sundaram, S., Retnakumari, A.P. and Anto, R.J. 2020. Pre-clinical evidences for the efficacy of tryptanthrin as a potent suppressor of skin cancer. *Cell Proliferation*, **53**(1): e12710. [*https://doi.org/10.1111/cpr.12710*].

Sreeja Devi, P.S., Kumar, N.S. and Sabu, K.K. 2021. Phytochemical profiling and antioxidant activities of different parts of *Artocarpus heterophyllus* Lam.(Moraceae): A review on current status of knowledge. *Future Journal of Pharmaceutical Sciences*. **7**: 1-7.

Srivastava, R. 2014. A review on phytochemical, pharmacological, and pharmacognostical profile of *Wrightia tinctoria*: Adulterant of Kurchi. *Pharmacognosy Reviews*, **8**(15): 36. [*doi: 10.4103/0973-7847.125528*].

Srivastava, R., Mukerjee, A. and Verma, A. 2015. GC-MS analysis of phytocomponents in pet ether fraction of *Wrightia tinctoria* seed. *Pharmacognosy Journal*, **7**(4). [*doi:10.5530/pj.2015.4.7*].

Vedhanarayanan, P., Unnikannan, P. and Sundaramoorthy, P. 2013. Antimicrobial activity and phytochemical screening of *Wrightia tinctoria* (Roxb.) R. Br. *Journal of Pharmacognosy and Phytochemistry*, **2**(4): 123-125.

Yadala, K.P., Syed, A. and Jalaja, N. 2020. Evaluation of phytochemicals, antimicrobial, antioxidant and DNA damage protection activity from in-vitro culture extracts of *Wrightia tinctoria* (Roxb.). *Plant Cell Biotechnology and Molecular Biology*, **3**: 71-81.