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RESEARCH ARTICLE

Molecular Characterization, Phytochemical Analysis, Antioxidant and Anticancer Potentials of *Wrightia tinctoria*

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ABSTRACT

In worldwide the use of the plant materials to prevent and treat infectious diseases successfully over the years has attracted the attention of scientists. Plant based anticancer/antioxidant compounds can serve the purpose without any side effects often associated with synthetic drugs and also little chance of development of resistance. The importance of the antioxidant constituents of plants in the protection from dreadful disease like cancer is also raising interest among scientists. In the current study the phytoconstituents in the *Wrightia tinctoria,* plant leaf extracts were analysed and estimated using qualitative and quantitative phytochemistry. The scavenging activity (antioxidant activity) was determined using DPPH method and it was found that water extract had the highest antioxidant activity, i.e., 27.14% while for methanol extract, it's the least, i.e., 9.72% and for chloroform extract, it is 19.27%. MTT assay was performed to understand the cytotoxicity of the extracts against liver cancer cell lines HepG-2.The cancer cells were treated with methanol extract at three different concentrations of 100µg, 500µg and 1000µg. The result obtained was then compared with control and positive control (cancer cells were treated with anticancer drug- Cyclo-90) and it was found that at the concentration of 1000µg, anticancer activity was highest and effective and the percentage toxicity was found to be 76.54% which is very close to that of Cyclo-90 drug, i.e., 77.74%. This leads to the conclusion that, due to its highly important medical nature was then subjected to molecular characterization to generate a DNA barcode. The matK gene was used as a marker and was amplified by PCR. The amplicons were subjected to DNA sequencing and the sequences so obtained were subjected to sequence alignment using BLAST tool. From this, *Wrightia tinctoria* has been identified to possess the anti-cancer property and can be used for curing cancer.

Keywords: *Wrightia tinctoria,* antioxidant, anticancer, phytochemical, DNA barcoding.

INTRODUCTION

Medicinal plants are gift of nature to cure a number of ailments of human beings. With the onset of scientific research in herbals, it is becoming clearer that the medical herbs have a potential in today's artificial era, as large number of medicines are becoming resistant. According to one estimate, only 20%of the plant flora has been studied and 60% of synthetic medicines be indebted their origin from plants. Ancient knowledge coupled with scientific principles can come to the forefront and provide us with powerful remedies to eradicate the various diseases. Extracts of higher plants have served as good quality sources of antibiotics against various bacterial and fungal pathogens [1].

The common view in the society and the medical community is that plant based products are healthier, safer and more reliable than synthetic products , even though safety and efficacy data are available for only a few number of plant materials. Plants act generally to stimulate and supplement the bodies healing forces; they are the natural foods of human beings [2]. *Wrightia tinctoria* plant leaves are very useful in the fever, intestinal worms, dysentery. The bark and seeds are effective against psoriasis and non-specific dermatitis. Anti dandruff properties of this plant acts as a basis for the hair oil preparations.

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Phytochemicals are plant-derived chemical compounds having potential health-promoting properties. Antioxidants are radical scavengers which help to protect the human body against free radicals that may cause pathological conditions such as ischemia, anemia, asthma, arthritis, inflammation, neuro-degeneration, Parkinson's disease, mongolism, ageing process, dementias and perhaps cancer [3]. Plants have long been used in the treatment of cancer [4]. The National Cancer Institute collected about 35,000 plant samples from 20 countries and has screened around 114,000 extracts for anticancer activity [5].

Selecting anticancer compounds including human tumor stem cell assay, human tumor *in vitro* cell line screening, hollow fiber assay, and human tumor xenografts [6]. Among them, only human tumor *in vitro* cell line screening has been widely used in primary drug screening. This approach typically uses a sulforhodamine B-based *in vitro* assay to test the toxicity of potential compounds against 60 different human tumor cell lines [7]. A similar approach is to use LDH or MTT assay to determine the toxicity of the tested samples on cells [8]. The identification of species depends on the knowledge held by taxonomists whose work cannot cover all taxon identification requested by non specialists. To deal with these difficulties, the 'DNA Barcode of Life' project aims to develop a standardized, rapid and inexpensive species identification method accessible to non-specialists (i.e. non-taxonomists). A combination of the non-coding *trnH-psbA* spacer region and the coding *rbcL* gene was a two-locus global barcode for land plants. In this present studies qualitative and quantitative analysis of phytochemical performed on *Wrightia tinctoria* leaf extracts. Scavenging activity was determined by DPPH assay. MTT assay was used to examine anticancer activity by using liver cell line HepG-2. Molecular characterization of the plant was performed by amplifying Mat K gene. The amplicons were sequenced and analyzed using BLAST tool [9].

MATERIALS AND METHODS

Sample Collection

The *Wrightia tinctoria* leaves were collected from Guindy, Chennai.

Extract Preparation

Leaves of *Wrightia tinctoria* were collected and it was shade-dried for ten days and reduced to course powder by using a mortar and pestle. The powdered leaves (10gm/100ml) were subjected to successive solvent extraction with the solvents (methanol, chloroform, water).The finally powdered leaf materials were put in different solvents- methanol, chloroform, aqueous. Methanol and chloroform solvents were kept in a shaker for over-night incubation and water solvent was kept in water bath for 1hr and then filtered. They are then subjected to evaporation under sunlight. Extract remains after solvent evaporation [10].

Qualitative Phytochemical Analysis:

The various qualitative chemical tests were carried out by using standard procedures described by Software (1993) [11].

Quantitative Phytochemical Analysis

Determination of Flavonoid:

10mg of dried sample is taken in a falcon tube and 10ml of respective solvent was added and dissolved. To 0.5ml sample (10mg dried extract per 10ml respective solvent), added 1.5ml methanol, 0.1ml of 1% $AlCl_3$, 0.1ml of 1N Potassium acetate and 2.8ml DW. The mixture was incubated for 30minsat room temperature and O.D. at 415nm was taken.

Determination of Carbohydrate:

To 1gm of powdered sample, added 2ml DW and grind in mortar and pestle. Then 13ml more DW was added and incubated for 20mins. The mixture was transferred into Falcon tubes and centrifuged at 10000rpm for 12mins. The supernatant was stored. The amount of carbohydrate was determined using Anthrone method.

Determination of Lipids

To 1gm of sample, added 6ml Petrolium ether and left overnight. Then incubated for 1hr at 40°C in water bath and filtered. An empty eppendorf tube was weighed. Then it was filled with the filtrate and kept for drying. Eppendorf tube with dried filtrate was weighed.

Determination of Protein

To 1gm sample, added 5ml 1X PBS and ground in mortar and pistle. Transferred to falcon tube and the volume was made up to 12ml. Incubated for 20min and centrifuged at 10000rpm for 10min and the supernatant was stored in deep freezer to which the Lowry method was performed.

Antioxidant/Free Radical Scavenging Activity

All the three extracts were used for determination of antioxidant activity. 50µl of standard and the extracts (1mg/ml) were dissolved in 3ml methanol and mixed with 10µl of DPPH solution. The mix was vortexes and incubated for 30 minutes inside UV Spectrophotometer so that the optical density of the solution can be measured for every minute. The DPPH radical scavenging activity was

calculated from the absorption according to the following equation.

$$\text{Radical scavenging activity}(\%) = [(\text{OD control} - \text{OD sample}) / \text{OD control}] \times 100$$

Where, the OD sample represents absorption of the sample solution, and OD control is for the control solution (not containing the sample). The radical scavenging activity is represented as percentage inhibition of DPPH radical.

Thin Layer Chromatography

10mg of dried sample was dissolved in 250µl of respective solvent. Solvents for running plate-chloroform: methanol (9:1) the data was analyzed and Rf values were calculated for the bands obtained.

MTT Assay on Hepg2 Cell Lines

The cells were grown in a 96-well plate in Delbucco's Minimum essential *medium* (DMEM) (HiMedia) supplemented with 10% fetal bovine serum (Gibco Laboratories) and antibiotics (streptomycin, penicillin-G, kanamycin, amphotericin B). About 1 ml cell suspension (10^5cells/ml) was seeded in each well and incubated at 37^0 C for 48 hour in 5% CO_2 for the formation of confluent monolayer. The monolayer of cells in the plate was exposed to various dilutions of the extract. The cell viability was measured using MTT assay with MTT (5 mg/ml) and DMSO. This tetrazolium salt is metabolically reduced by viable cells to yield a blue insoluble Formosan product measured at 570nm spectrophotometerically (Kang et al., 2004). Controls were maintained throughout the experiment (untreated wells as cell control). The assay was performed in triplicate for each of the extracts. The mean of the cell viability values was compared to the control to determine the effect of the extract on cells and % cell viability was plotted against concentration of the plant extract

Molecular Characterization:

Isolation and quantification of DNA

Isolation of genomic DNA followed the procedure of Doyle and Doyle method (1990) [12].Isolated DNA observed on agarose gel electrophoresis and quantified by spectrophotometric method. A solution of 40µg/ml concentration of single stranded DNA in a quartz cuvette corresponds to 1 when measured at 260nm. The ratio of absorbance at 260nm and 280 nm provides a clear idea about the purity of the DNA sample The absorbance at 260nm and 280 nm was set at zero using the blank as TE buffer.3µl of DNA was taken in the cuvette and made up to 3ml using TE buffer. The absorbance at 260nm and 280nm the spectrophotometer was calibrated and the wavelength was set as 260nm and 280 nm. were observed. The concentration of the DNA in the sample was calculated using the formula:

Concentration of DNA=A_{260}*50µg/ml*dilution factor.

PCR and BLAST

PCR is an invitro method of enzymatic synthesis of a specific DNA sequence developed by Kary Muller PCR was done using matK primer [13]. PCR product was eluted by gel elution method and PCR ampilicon was sequenced by Sanger's method using 3730 DNA sequencing analyzer at ABI. The amplicons were then subjected to nucleotide sequencing, which yielded the gene sequence. The obtained sequence when analyzed using BLAST tool, showed similarity to the matK gene sequence of Wrightia tinctoria plant available in the database.

RESULTS &DISCUSSION

Wrightia tinctoria is a widely used plant, traditionally in our alternative system of medicinal practice for the treatment of skin infection. Now with the advent of newer antibiotics and new infectious diseases we are observing development of multi drug resistance by organisms to different antibiotics. But *Wrightia tinctoria* is such a plant which is used since time immemorial for skin infection and still effective. Phytochemicals mixture present in different solvents as shown in the result of phytochemical screening might be knowledgeable to the ability of the solvents to dissolve into solution specific type of Phytochemicals. Methanol leaf extract showed the presence of flavonoids, phenolics and steroids. Ethanol extract shown the presence of flavonoids, phenolics, steroids and tannins in the leaf extract of *Wrightia tinctoria*. [14]

The present study qualitative phytochemical analysis was performed on three samples of *Wrightia tinctoria* leaf extracts obtained by solvents methanol, chloroform and water and it was found that carbohydrate was present in all the three extracts while flavonoids were found to be present only in methanol extract showed in the table: 1.

Table: 1 Qualitative Analysis Of Phytochemical

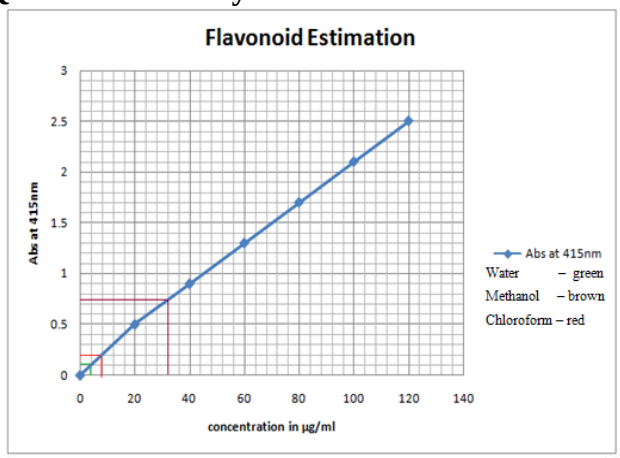
Phytochemicals	Methanol	Chloroform	Water
Carbohydrates	Present	Slightly present	Present
Tannins	Present	Present	Slightly present
Saponins	Absent	Absent	Slightly present
Flavonoids	Present	Absent	Absent
Alkaloids	Present	Absent	Slightly present

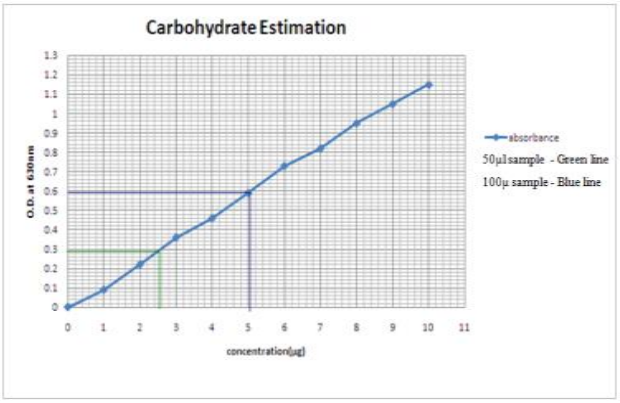
Cyanins	Absent	Absent	Absent
Quinones	Present	Absent	Absent
Glycosides	Present	Absent	Absent
Cardiac Glycosides	Absent	Absent	Absent
Terpenoids	Absent	Absent	Absent
Triterpenoids	Absent	Slightly present	Slightly present
Coumarins	Slightly present	Absent	Absent
Phenols	Present	Present	Present
Acids	Absent	Absent	Absent
Amino acids	Absent	Absent	Absent

Quantitative Analysis

Quantitative analysis for flavonoids, carbohydrates and proteins was also performed. According to flavonoids estimation, it was found to be highest in methanol extract, i.e., 12.2µg/ml while in water extract, its only 4.2µg/ml and in chloroform extract, its 8 µg/ml. From carbohydrate estimation, it was found to be present at a concentration of 765 to 780µg per gram of dry leaves. After calculation, protein concentration was found in the range of 1.560 to 1.824gm per gram of dry leaves showed in the fig: 1.

Quantitative Analysis





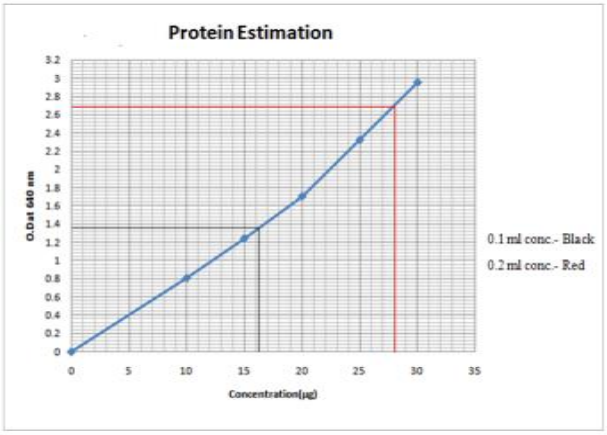


Fig: 1 Quantitative Analysis of flavonoid, carbohydrate and protein

Scavenging Activity

Wrightia tinctoria R. Br. is considered to be very effective jaundice plant in Indian indigenous system of medicine [15]. Plant is considered to have higher antioxidant activity and can be used for the treatment of liver cancer cells. Many compounds of plant origin have been identified as that inhibits different stages in the replication cycle of HIV [16].In this study scavenging activity was determined using DPPH method and it was found that water extract had the highest scavenging/antioxidant activity, i.e., 27.14% while for methanol extract, i.e., 11.12% and for chloroform extract, its 19.27% showed in the table 2 & fig 2.

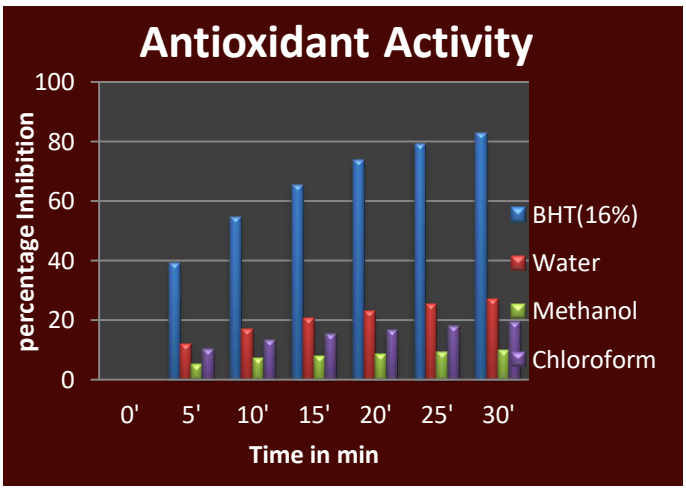


Table: 2 Percentage Inhibition of Scavenging activity

Time(min)	0'	5'	10'	15'	20'	25'	30'
BHT(16%)	0	38.93	54.5	65.57	73.77	79.3	82.58
Water	0	12	17.14	20.57	23.14	25.43	27.14
Methanol	0	5	8.22	8.65	9.31	9.77	11.12
Chloroform	0	10.24	13.17	15.12	16.58	18.05	19.27

Fig : 2 Graphical representation of Antioxidant activity in all the three samples in comparison to the Standard Antioxidant BHT(16%).

Thin Layer Chromatography

The difference in Rf values reflected the qualitative variation in the phytocompounds. In one study, the HPTLC analysis of methanol leaf

extract of *Wrightia tinctoria* and *Wrightia coccinea* recorded the presence of indole and flavonoid constituents [17].

Thin Layer chromatography was also being performed for the three extracts to obtain chemical profile for the plant. According to TLC data analysis, from water extract, no bands were obtained while for methanol extract, four bands were obtained with Rf values 0.31, 0.41, 0.53 and 0.69 respectively and for chloroform extract, only two bands were visible with Rf values of 0.53 and 00.67 showed in the fig: 3.

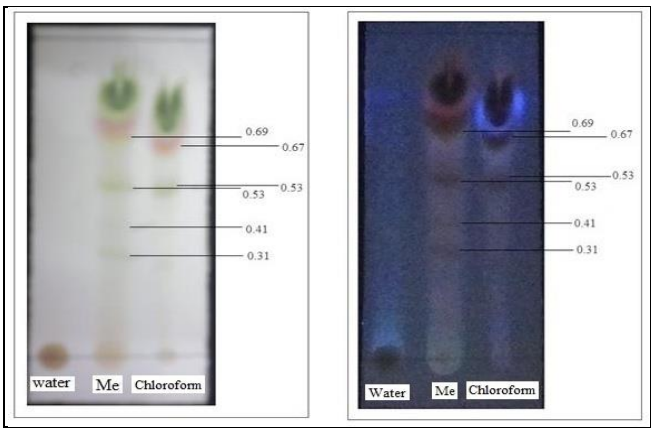


Fig: 3 Thin Layer Chromatography

MTT Assay

The effect of methanol leaf extract of *Wrightia tinctoria* plant on the growth of the HeLa cell line was examined by MTT assay. The extract was screened for its cytotoxicity activity at various concentrations to determine the IC50 (50% growth inhibition) value. As the concentration increased from 1.23-100µg/ml, percentage of inhibition increases from 6.93%-47.53%. The IC50 value was found to be 71.6µg/ml from the non-linear regression equation [18].

Anticancer activity was examined by MTT assay method using liver cancer cell line- HepG-2. As flavonoids were found in higher concentration in methanol extract, the cancer cells were treated with methanol extract at three different concentrations of 100µg, 500µg and 1000µg. The result obtained was then compared with control and positive control (cancer cells were treated with anticancer drug- Cyclo-90) and it was found that at the concentration of 1000µg, anticancer activity was highest and effective and the percentage toxicity was found to be 76.54% which is very close to that of Cyclo-90 drug, i.e., 77.74%showed in table: & fig: 4.

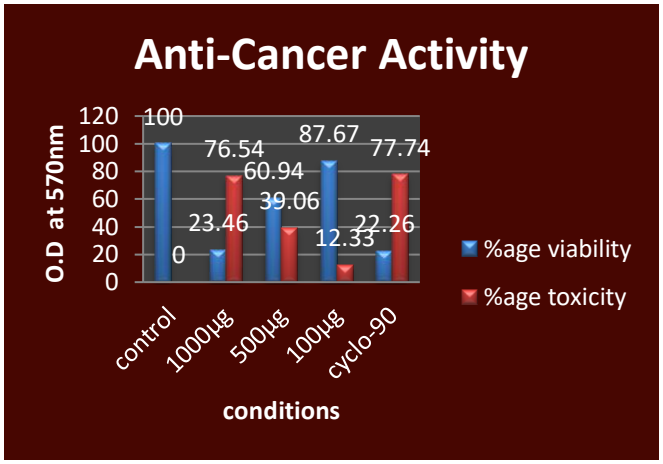


Fig: 4 Graphical representation of estimated Anticancer activity.

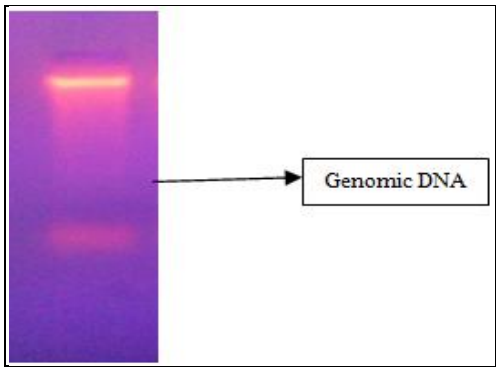
Table 3 percentage viability and toxicity.

Control	Condition	%age viability	%age toxicity
1.833	1.833	100	0
1.833	0.43	23.46	76.54
1.833	1.117	60.94	39.06
1.833	1.607	87.67	12.33
1.833	0.408	22.26	77.74

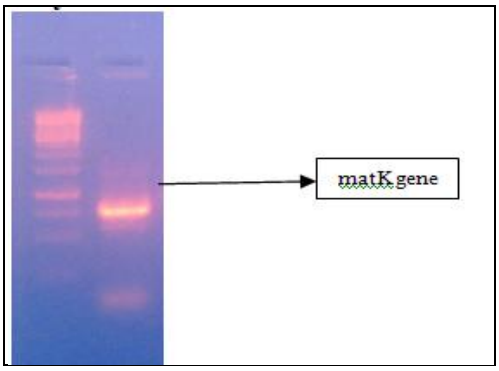
Molecular Characterization

Keeping the high medicinal importance of the plant leaves, molecular characterization of the plant was performed by amplifying the matK gene, which is a conserved region among all plants and is usually used as marker gene. The genomic DNA was isolated and quantified. PCR was done using matK primers. The amplicons were then subjected to nucleotide sequencing, which yielded the gene sequence. The obtained sequence when analyzed using BLAST tool, showed similarity to the matK gene sequence of Wrightia tinctoria plant available in the database.

Genomic Dna Isolation



Polymerase Chain Reaction for matK



Lane 1: 1kb DNA Ladder (10,000 bp, 8000 bp, 6000 bp, 5000 bp, 4000 bp, 3000 bp, 2500 bp, 2000 bp, 1500 bp, 1000 bp, 750 bp, 500 bp, 250 bp)

Lane 2: Amplified matK from plant

Molecular weight: 1500 base pair (bp)

>Sequence

TTCTACGCGAGTATTGTAATTGCAATAAA
ATTATTGCTACAAAGAAACCCGGTTTTCAT
TTTTTAACAAAAAGAAATCAAAGATTATT
CTTCTTCTTATATAATTTTTATGTATGTGA
ATACGAATCCATTTTCGTCTTTCTCCATAA
CCAATCTTCTCATTTACGATCAACATCCTT
TGGGGTCCTTCTTGAACGAATCTATTTCTA
TGGAAAAATAGAACGTCTTGTCGACGTCT
TCGCTAAGTTTTTTCAGACCAACTTATGCT
TGTTCAAATATCCTTTCATGCATTATGTTA
GGTATCGATTACGTTTCAAGGGGGACGCC
TCTTTTGATGAATAAATGGAAATCTTACCT
TGCCAATTTTTGGCAATGTAATTTTGACCT
GTGGTTTCACTTGGAAAGGGTCTATATAA
AGCAATTGTCCAATCATTCCCTTGATTTTA
TGGGTTATCTTTCAATTGTGCGACTAAATA
TATCATGCATCGGCTCGAAAATGCATTTCT
GATTAATAATGCTATTAAGAAATTCGATA
CCCTTGTTCCAATTATTCCTCTGATTGGAT
CATTGGCTAAAGCGAAATTTTGTAACCTAT
TAGGACATCCCGTTAGTAAACCGGTTCGG
ACTGATTTATCAGATTCTGATATTATGGAC
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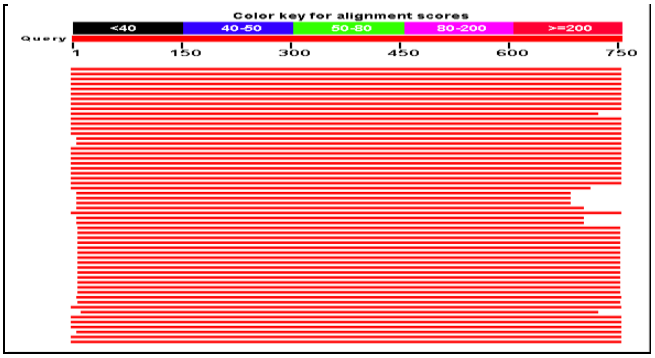

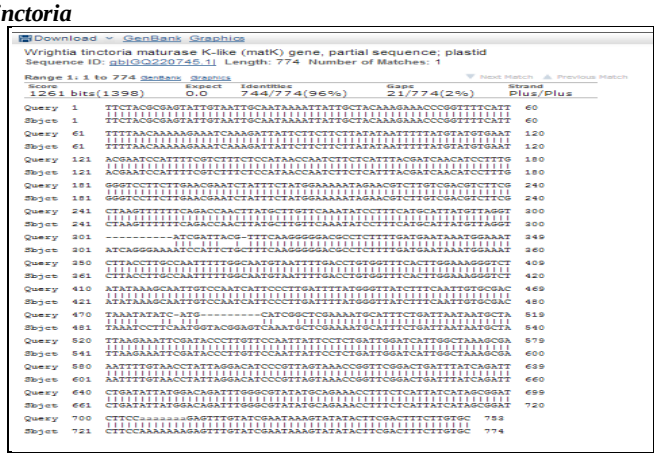

CONCLUSION

Wrightia tinctoria are high in bioactive compounds like phenolic and flavonoid content. Leaf extract of *Wrightia tinctoria* showed the remarkable antioxidant activity and also anticancer property to control the progression of cancer.This study evaluated the importance of anticancer property of *Wrightia tinctoria* leaf extract, in future it will become a natural drug for cancer.

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