https://africanjournalofbiomedicalresearch.com/index.php/AJBR
Afr. J. Biomed. Res. Vol. 27(3s) (September2024); 1472 - 1481
Research Article

In Vivo And *In Vitro* Study On Anticancer Activity Of Methanolic Extract Of *Cryptostegia Grandiflora* Leaves In Experimental Animals

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Abstract:
Objective: To assess the *in vivo* and *in vitro* study on anticancer activity of methanolic extract of *Cryptostegia grandiflora* leaves in experimental animals.
Methods: Methanolic extracts of *Cryptostegia grandiflora* is used for *In vitro* anticancer activity by MTT assay and in vivo against sarcoma growth in a mouse model. Also we used instrumental analysis such as Column Chromatography, UV, HPTLC, GCMS and ^{1}H NMR.
Results: sample was performed for *In vitro* anticancer activity the compounds Sample GN showed less toxic activity against normal fibroblast cell line when you compared to standard drug 5 FU using MTT assay. Subfractions 1 and 2 significantly inhibited tumor growth in mice bearing Sarcoma-180 cells as compared with the control mouse tumor, and subfraction 2 showed greater inhibition of tumor growth than did subfractions 1 with our *in vivo* results.
Conclusions: CGME has contributed and to show *in vivo* and *in vitro* anticancer activity from the animal study we observed that toxicity is not occurs and all the data related to the pharmacological activity was statistically analyzed.

Keywords: *Cryptostegia grandiflora*, Anticancer activity, Methanolic extract, *In vitro and In vivo* activity.

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Submitted- 05/July/2024 Reviewed- 13/August/2024 Accepted- 18/September//2024 Published- 30 September/2024

DOI: https://doi.org/10.53555/AJBR.v27i3S.2335

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Introduction:

Cryptostegia grandiflora, commonly known as rubber vine, is a plant native to tropical regions and has been traditionally used for various medicinal purposes. Recent studies have shown that plants belonging to the Apocynaceae family, which includes *C. grandiflora*, possess significant biological activities, including antioxidant and anticancer properties.[1] The increasing interest in natural antioxidants from plants is due to their ability to neutralize free radicals and prevent oxidative stress, which is linked to various chronic diseases, including cancer.[2]

Cryptostegia grandiflora, commonly known as rubber vine, is a woody-perennial vine that is native to south-west Madagascar. It has also been introduced to most other tropical and subtropical regions by man, because

of its attractive flowers and the fact that its latex contains commercial quality rubber (hence the name). It is very similar to the purple rubber vine (C. madagascariensis),which is also native to Madagascar.[2] Nature always stands as golden mark to amplify the outstanding phenomenon of symbiosis.

This study aims to explore the anticancer potential of the methanolic extract of *C. grandiflora* leaves using both in vitro and in vivo models. We hypothesize that the phytochemical constituents present in CGME contribute to its biological activities, which could be beneficial in the development of new therapeutic agents.[3]

Materials and Methods:

Plant Collection and Extraction: The aerial parts of *Cryptostegia grandiflora* were collected from Atpadi District, Sangli, Maharashtra, India, and authenticated by a botanist. The plant material was shade-dried, powdered, and subjected to extraction using methanol in a Soxhlet apparatus. The methanolic extract was concentrated under reduced pressure to obtain a crude extract, which was then stored at 4°C for further use.

***In vitro* cell viability assay**

Principle of assay: This Colorimetric assay is based on the capacity of Mitochondria succinate dehydrogenase enzymes in living cells to reduce the yellow water soluble substrate 3-(4, 5- dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into an insoluble, purple colored formazan product which is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells.

Experimental procedure:[4-5]

1) L929 Normal fibroblast cell line was procured from National center for cell sciences (NCCS), Pune maintained in DMEM medium supplemented with 10 % fetal bovine serum.
2) Cells were incubated at a concentration of 1 × 104cells/ml in culture medium for 24 h at 37°C and 5% CO2.
3) Cells were seeded at a concentration (70µl) 104cells/well in 100µl culture medium and 100µl Sample GN (25 ,50, 100 µL) into micro plates respectively (tissue culture grade, and 96 wells).
4) Control wells were incubated with DMSO (0.2% in PBS) and cell line. All samples were incubated in triplicate. Controls were maintained to determine the control cell survival and the percentage of live cells after culture.
5) Cell cultures were incubated for 24 h at 37°C and 5% CO2 in CO2 incubator (Thermo scientific BB150)
6) After incubation, the medium was completely removed and Added 20 µl of MTT reagent (5mg/min MTT Formazan PBS).
7) After addition of MTT, cells incubated for 4 hrs at 37oC in CO2incubator.
8) Observed the wells for formazan crystal formation under microscope. The yellowish MTT was reduced to dark coloured formazan by viable cells only.
9) After removing the medium completely. Added 200µl of DMSO (kept for 10 min) andincubate at 370C (wrapped with aluminium foil).
10) Triplicate samples were analyzed by measuring the absorbance of each sample by a Elisa microplate reader (Benesphera E21) at a wavelength of 570 nm.

***In vivo* anticancer assays[6-8]**

Anticancer activity *in vivo* against sarcoma growth in a mouse model was examined using the method with minor modification.[6-8] Briefly, Balbc/c mice (six-to seven-weeks-old) randomly divided into the control and six experimental groups, and provided with water and normal chow.

Animals were housed in an air-conditioned room at 21-26°C with 45-55% moisture and a 12- hour dark/light cycle. The Sarcoma-180 cell line wascultured for 7-10 days in the abdominal cavity of a Balbc/c mouse, and the cultured cells were harvested with the peritoneal fluid and centrifuged at 1,200 rpm for 10 min in phosphate-buffered saline (PBS). The separated sarcoma cells were floated in PBS and centrifuged, and their concentration was adjusted to 1.0×10^6 cells/ml. The cells (0.2 ml) were implanted subcutaneously in the left groins of mice. After 24 h, the mice were fed normal chow supplemented with water (control group), 0.1 mg subfraction 1 per mouse per day (S-180+subfraction 1A), 0.2 mg subfraction 1 per mouse perday (S-180+subfraction 1B), 0.1 mg subfraction 2 per mouse per day (S-180+subfraction 2A),

0.2 mg subfraction 2 per mouse per day (S-180+subfraction 2B), 0.1 mg subfraction 3 per mouse per day (S-180+subfraction 3A) or 0.2 mg subfraction 3 per mouse per day (S-180+subfraction 3B) for 20 days. The mice were fed normal chow for 7 days after the last oral administration of subfractions and then euthanized with an ip injection of Avertin [91] on the 27th day after cancer- cell implantation

Instrumental Analysis: CGME was subjected to instrumental analysis for compound identification. Column Chromatography was performed to isolate fractions, followed by UV spectrophotometry, HPTLC, GCMS, and 1H NMR to identify and confirm the structure of bioactive compounds.[9-10]

Results:

Extraction and standardization:

Table No: 1. Macroscopic Characteristics of leaves of *Cryptostegua grandiflora Linn Roxb*

Sr. No.	Parameters	Observation of leaves
1	Colour	Greyish green
2	Odour	Odourless
3	Taste	Bitter
4	Size	1.2- 2.0 cm in diameter
5	Shape	Globular or oval
6	Surface	Smooth & shiny

Table No. 2: Standardization of leaves *Cryptostegia grandiflora* Linn Roxb

S. No.	Physical Contents	Result
1	**Extractive Values (% w/w)**	
	Ethanol soluble extractive	9.50
	Methanol soluble extractive	12.56
2	**Loss on Drying (% w/w)**	**8.20**
3	**Ash Value (% w/w)**	
	Total Ash	4.75
	Acid Insoluble Ash	0.95
	Water Soluble Ash	1.90

Table No: 3. Solvent extraction of air-dried plant material of *Cryptostegia grandiflora Linn.* Roxb.

Sr. No.	Extracts	Nature of Extract	Colour	Weight (g) %	Yield (w/w)
1	Ethanol	Semi-solid	Greyish Green	24.50	9.50
2	Methanol	Semi-solid	Greyish Green	26.50	12.56

Fig. 1 Leaf soxhlet extraction of *Cryptostegia grandiflora Linn Roxb leaves extract.*



Coarsely powdered *Cryptostegia grandiflora Linn Roxb* leaves were extracted with ethanol & methanol extract using soxhlet apparatus.

PHYTOCHEMICAL INVESTIGATION

a Qualitative Chemical Analysis:

Table 4 : Qualitative chemical test for extract of *Cryptostegia grandiflora Linn Roxb*

Sl. No	Photochemical constituents	ECG	MCG
1	Cardiac Glycoside (Baljet test)	+	+
2	Alkaloids(Mayer's reagent)	+	+
3	Flavonoids (Shinoda test)	+	+
4	Tannins	+	+
6	Saponin: (Foam test)	-	+
7	Carbohydrate: (Molisch test)	+	+

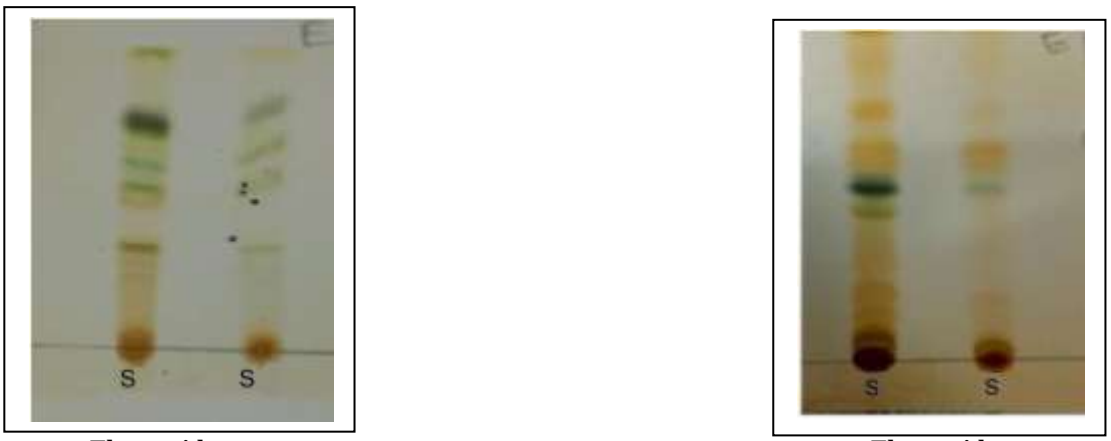
Photochemical analysis of different extract of CG-leaves was done as shown in method section.
ECG=Ethanol *Cryptostegia grandiflora;*
MCG=Methanol *Cryptostegia grandiflora*,(+) Present (-) Not detected.

The results of qualitative chemical investigate ion of various extracts of whole plant of *Cryptostegia grandiflora* leaves. Have indicated the presence of the following compounds:

✓ **Methanol extract:** Carbohydrates, Flavonoids, Tannins and Saponin compounds.

b Chromatography techniques TLC of *Cryptostegia grandiflora*

The extract was subjected to TLC has shown the presence of 4 isolated spots with iodine vapours and UV (278 nm).



Flavonoids **Flavonoids**

Ethanol: Methanol (5:5) **Benzine: Methanol(95:5)**

Fig. 1. TLC for Methanol showing the separation of Flavonoids.

c. Column Chromatography

Column chromatography gave different fractions and these fractions were concentrated and their purity was determined by using thin - layer chromatography. Retention factor is the distance moved through the stationary phase to that of mobile phase.



Fig. 2. *Cryptostegia grandiflora* extraction separation by using column chromatography

Table 5: Column chromatography (R f values and weight % for isolated compounds) of Cryptostegia grandiflora

FRACTIONS	Rf VALUES	% WEIGHT
Component 1	0. 52	0.06
Component 2	0. 30	0.08
Component 3	0. 43	0.05
Component 4	**0.37**	**0.05**
Component 5	0.71	0.04
Component 6	0.54	0.07
Component 7	0.47	0.09

The isolated components were designated as Q1, Q2, Q3, Q4, Q5, Q6 and Q7 respectively. The isolated components, Q4 were confirmed as Flavonoid by qualitative chemical tests (Shinoda test).

d. UV

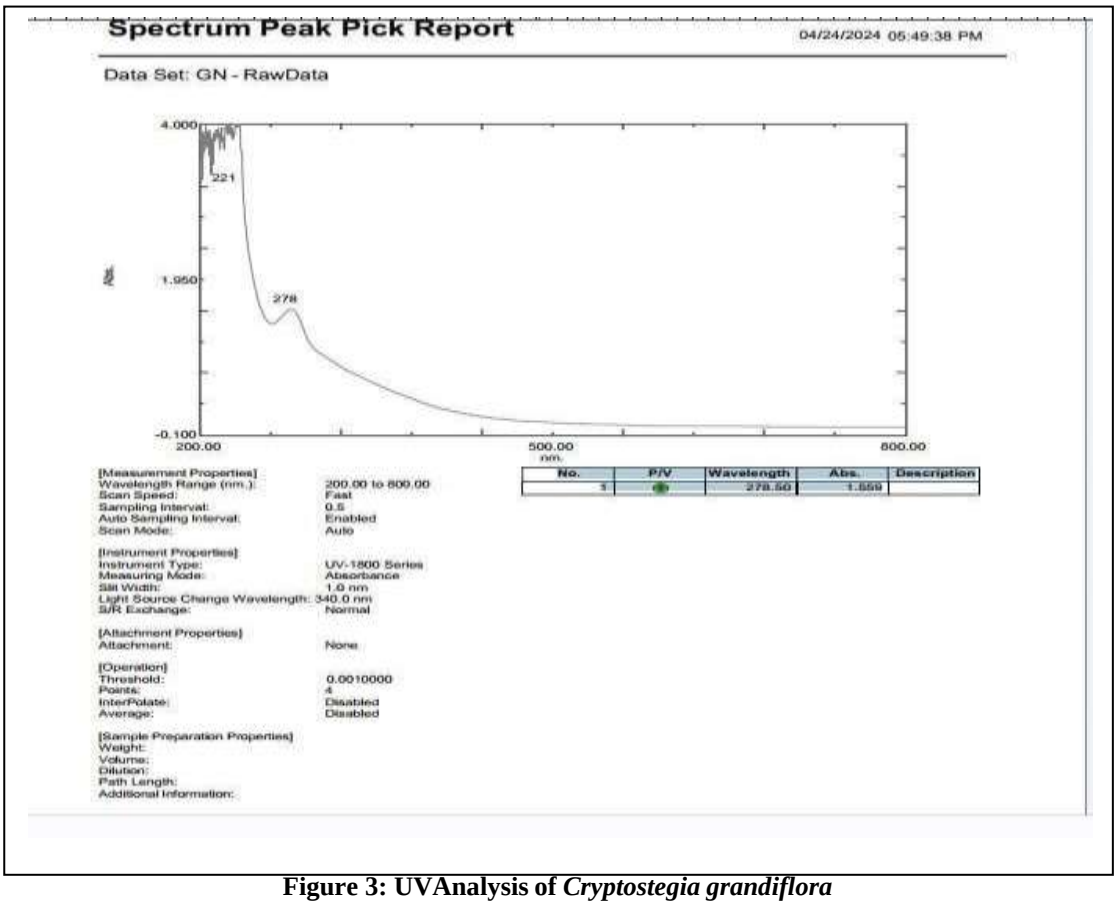


Figure 3: UVAnalysis of *Cryptostegia grandiflora*

e. HPTLC SCREENING

HPTLC study was carried out for the quantification of Luteolin in extract. Visualization was performed as done. After development the plate was scanned in densitometer under 254nm and the chromatogram obtained is depicted in figure 6.5.

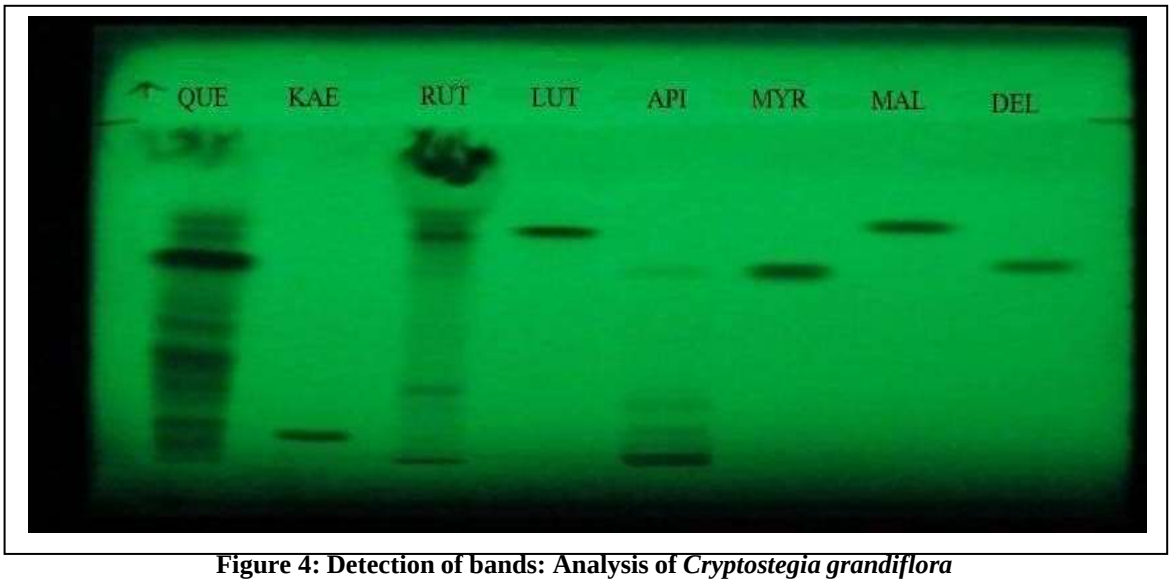


Figure 4: Detection of bands: Analysis of *Cryptostegia grandiflora*

Table 6: Lists of spots applied on HPTLC plate

Track number	Sample(5µL)
1	QUERCETIN
2	KAEMPFEROL
3	RUTIN
4	LUTEOLIN
5	APIGENIN
6	MYRICETIN
7	MALVIDIN
8	DELPHINIDIN

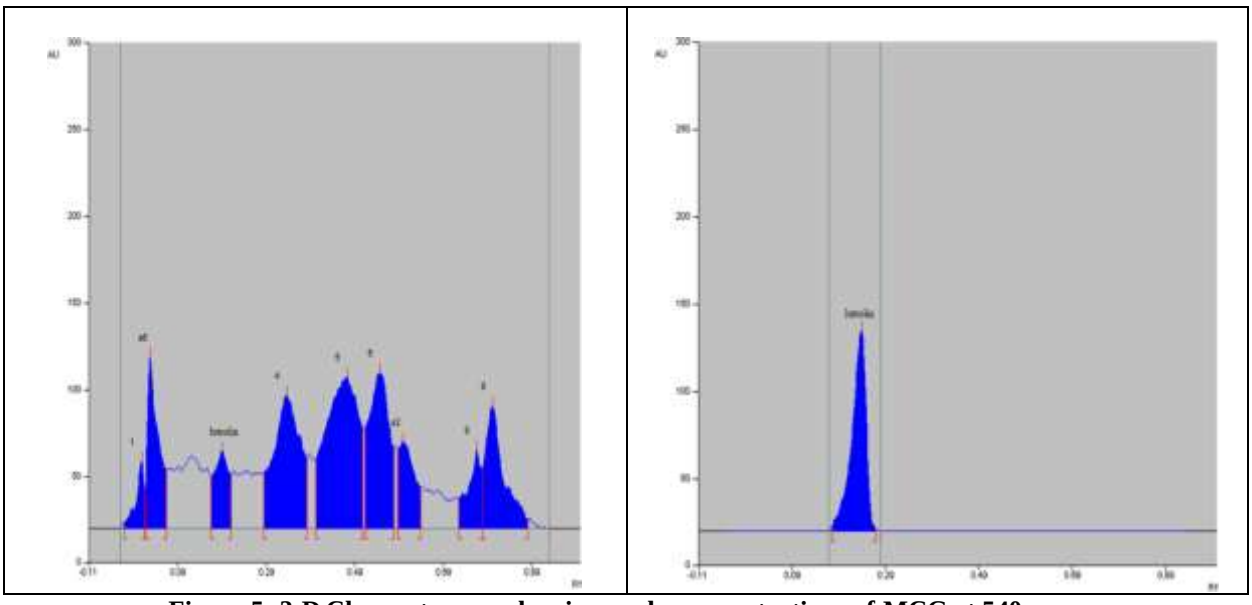


Figure 5: 3-D Chromatogram showing peaks concentrations of MCG at 540 nm

Table 7: Selection of wave lenght for Luteolin

Track	Application position	Application volume	Vial	Sample ID	Active
1	15.0 mm	0.2 µl	1	LUTEOLIN	
2	25.0 mm	0.4 µl	1	LUTEOLIN	
3	35.0 mm	0.6 µl	1	LUTEOLIN	
4	45.0 mm	0.8 µl	1	LUTEOLIN	
5	55.0 mm	1.0 µl	1	LUTEOLIN	
6	65.0 mm	1.2 µl	1	LUTEOLIN	
7	75.0 mm	0.4 µl	2	EXTRACT M	
8	85.0 mm	0.6 µl	2	EXTRACT M	

f. GC MS

The GC-MS analysis of *Cryptostegia grandiflora* showed GC fraction and fragmentation patterns of mass spectroscopy. The compounds were identified by GC-MS analysis. The mass spectra of the luteolin were compared with the library data of NIST and identified. Compound is identified in GCMS i.e. shown in figure 6.7.

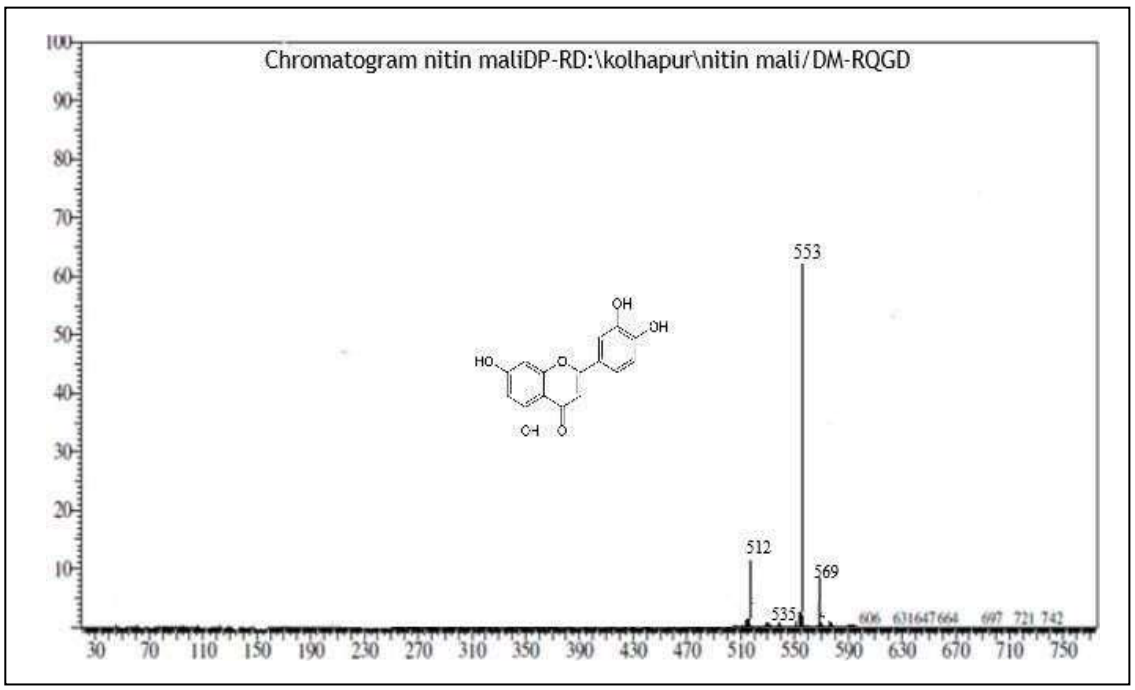


Figure 6: GC-MS analysis of *Cryptostegia grandiflora* extract as LuteolinTable 8: GCMS spectra of Luteolin

Sr. No.	Compound	Retention time	Molecular Weight	Molecular Formula	Concentration (%)
1	Luteolin	2.2923	286.24	C15H10O6	29

6.2. ^{1}H NMR

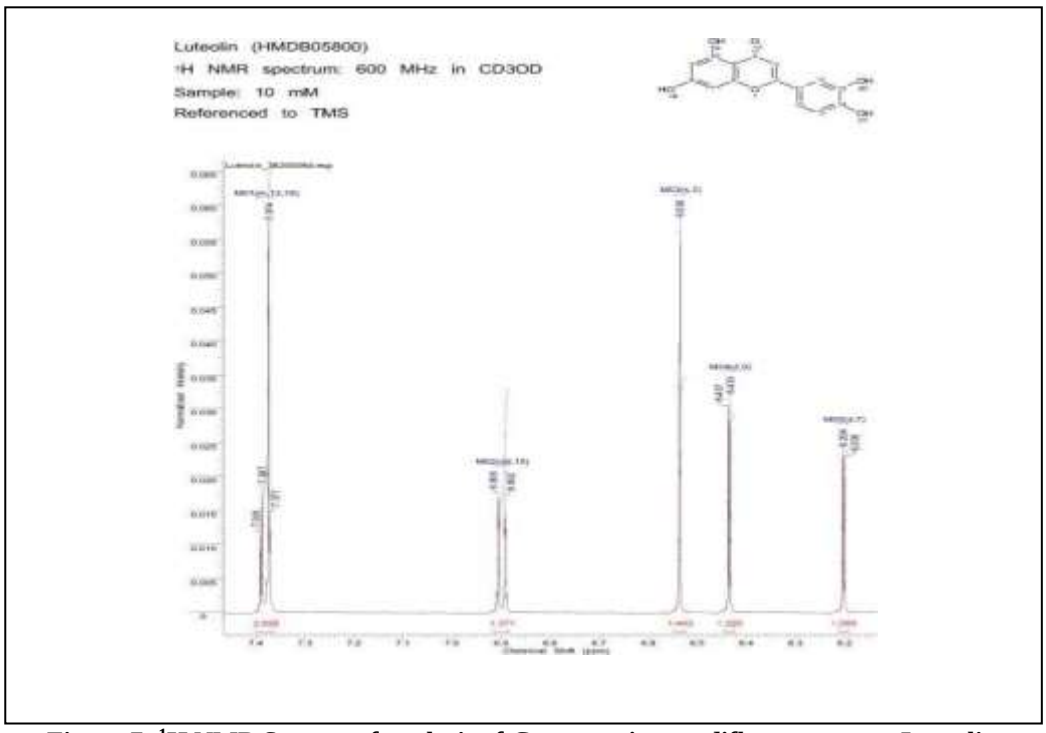


Figure 7: ^{1}H NMR Spectra of analysis of *Cryptostegia grandiflora* extract as Luteolin

Table 9: Table of Peaks

Sr. no	(ppm)	Height
1	8.20	0.0225
2	8.20	0.0232
3	8.43	0.0310
4	8.44	0.0298
5	8.89	0.0167
6	8.89	0.0139
7	8.90	0.0144
8	8.91	0.0170
9	7.37	0.0143
10	7.37	0.0165

***In vitro* Anticancer activity by MTT assay**

Table 10: *In vitro* cell viability assay

a) Sample Description	Sample- GN
b) Sample Form	Liquid
c) Activity	In vitro cell viability assay
d) Cell line	L929 Normal fibroblast cell line
e) Media	DMEM with high glucose (Cat No-11965- 092). FBS (Gibco, Invitrogen) Cat No – 10270106.Antibiotic – Antimycotic 100X solution (Thermo fisher Scientific)- Cat No- 1524 0062.

Table 11: Effects of Sample GN against L929 (normal fibroblast cell line) by MTT assay

Sr. No	Concentration (µg/ml)		Absorbance (OD)				Cell Viability (%)
			1	2	3	Average	
1	**Control**		1.265	1.312	1.254	1.277	
2	**Positive control-5FU**	20	0.426	0.359	0.325	0.37	28.97416

2	**GN 25**	25	0.857	0.865	0.825	0.849	66.48395
		50	0.822	0.831	0.815	0.822667	64.42182
		100	0.798	0.787	0.786	0.790333	61.88985

At the different doses (25 to 100 µg/ml) of Sample GN carried out for cell cytotoxicity activity against L929 normal fibroblast cell line. The compounds Sample GN showed less toxic activity against normal fibroblast cell line when you compared to standard drug 5 FU.

***In vivo* Anticancer activity**

Table 16: Antitumor activity of the three subfractions separated from *Cryptostegiagrandiflora* in Balbc/c mice bearing Sarcoma-180 cells (S-180)

	S-180 + water (control)	**S-180+ subfraction1A**	**S-180+ subfraction1B**	**S-180+ subfraction2A**	**S-180+ subfraction2B**	**S-180+ subfraction3A**	**S-180+ subfraction3B**
Tumor weight	4.01 ± 0.18^{e}	3.05 ± 0.13^{b}	2.62 ± 0.30^{a}	3.55 ± 0.15^{de}	3.25 ± 0.25^{bc}	3.40 ± 0.18^{ad}	3.08 ± 0.11^{b}
Inhibition rate	0.00 ± 4.55^{a}	22.95 ± 5.35^{d}	31.65 ± 8.30^{e}	6.60 ± 6.11^{ab}	17.40 ± 7.78	13.54 ± 5.63^{bc}	22.80 ± 3.19^{d}

Values are means ± SD of six mice.

Values with different superscripted letters (a-e) within the same row are significantly different ($P < 0.05$) as determined by Duncan's multiple-range test. The Sarcoma-180 cells were implanted subcutaneously in the left groins of mice. After 24 h, the mice were fed normal chow supplemented with water (S-180+water, control), 0.1 mg of subfraction 1 per mouse per day (S- 180+subfraction 1A), 0.2 mg of subfraction 1 per mouse per day (S-180+subfraction 1B), 0.1 mg of subfraction 2 per mouse per day (S-180+subfraction 2A), 0.2 mg of subfraction 2 per mouse per day (S-180+subfraction 2B), 0.1 mg of subfraction 3 per mouse per day (S-180+subfraction 3A) or 0.2 mg of subfraction 3 per mouse per day (S-180+subfraction 3B) for 20 days. The mice were fed normal chow for seven days after the last oral administration of subfractions. Solid tumors were removed on the 27th day after cancer cell implantation.

Conclusion:

Coarsely powdered *Cryptostegia grandiflora Linn Roxb* leaves were extracted with methanol extract using soxhlet apparatus. The results of qualitative chemical investigate ion of various extracts of whole plant *Cryptostegia grandiflora* compounds. The extract was subjected to TLC has shown the presence of 4 isolated spots with iodine vapours and UV (278 nm). Column chromatography gave different fractions and these fractions were concentrated and their purity was determined by using thin-layer chromatography. HPTLC study was carried out for the quantification of Luteolin in extract. Visualization was performed as done. After development the plate was scanned in densitometer under 254nm and the chromatogram obtained was depicted.

In case Sample GN carried out for cell cytotoxicity activity against L929 normal fibroblast cell line. The compounds Sample GN showed less toxic activity against normal fibroblast cell line when you compared to standard drug 5 FU. We hypothesize that the pure compounds (Luteolin) separated from Cryptostegia grandiflora would inhibit tumor growth in Balbc/c mice bearing Sarcoma-180 cells (S-180) in vivo test. In vivo results, subfraction 1 isolated from Cryptostegia grandiflora at concentrations of 0.1 and 0.2 mg/mouse per day significantly decreased tumor volume by 22.95% and 31.65%, respectively, as compared with the control. Subfractions 1 and 2 significantly inhibited tumor growth in mice bearing Sarcoma-180 cells as compared with the control mouse tumor, and subfraction 2 showed greater inhibition of tumor growth than did subfractions 1 with our in vivo results.

The results suggest that Cryptostegia grandiflora and its compounds in these subfractions isolated from Cryptostegia grandiflora could be used as natural anticancer ingredients in the food and/or pharmaceutical industry.

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