International Journal of Drug Development & Research
| January-March 2012 | Vol. 4 | Issue 1 | ISSN 0975-9344 |
Available online http://www.ijddr.in
Covered in Official Product of Elsevier, The Netherlands
SJR Impact Value 0.03,& H index 2

FULL Length Research Paper
Covered in Index Copernicus with IC Value 4.68 for 2010

Phytochemical analysis, Antioxidant and Cytotoxic activity of medicinal plant *Combretum roxburghii (Family:* Combretaceae)

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Abstract

Phytochemical analysis of *Combretum roxburghii* leaf and bark samples revealed presence of tannins, saponins and flavonoids. Hexane, dichloromethyl, chloroform, acetone and methanol solvent extracts of leaf and bark were tested for their antioxidant and cytotoxic activity. Positive antioxidant activity but with seasonal variation was found for all the extracts using TLC based standard qualitative 2,2 diphenyl-1-picrylhydrazys(DPPH) assay. However, a seasonal variation was observed in all the extracts. February and October were found to be favorable for the presence of antioxidant property. Acetone extract of *Combretum roxburghii* showed maximum antioxidant bands(25) and promising antioxidant activity at par with the standard antioxidant ascorbic acid(IC50 9.5microgram). Brine shrimp motility assay at the doses of 25 and 50microgram/ml showed variation in leaf extracts activity from 10 to 98%(February), 26-83%(May), 56-100%(July) and 86-100% in October. In comparison, activity of bark extracts varied differently 9-98%(February), 43-100%(May), 46-100%(July) and 23-100% for October samples. The potency of leaf extracts could be considered ideal in October where as for bark samples May and July months are significant. Acetone extract of the leaf showed 100 percent cytotoxic activity and was also rich in antioxidant properties, so same was fractionated and a pure antioxidant fraction was isolated which showed good antioxidant activity and moderate amount of cytotoxic activity in live cell assay using Jurkat cells (tumor cell line).

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Key words:
Antioxidant, cytotoxic, alkaloids, flavonoids, tannins, saponins

***How to Cite this Paper*:**
Sunita Bhatnagar*, Sunita Sahoo, Ajay Kumar Mohapatra and Dipti Ranjan Behera "Phytochemical analysis, Antioxidant and Cytotoxic activity of medicinal plant *Combretum roxburghii (Family:* Combretaceae)", Int. J. Drug Dev. & Res., Jan-March 2012, 4(1): 193-202

Article History:----------
Date of Submission: 26-12-2011
Date of Acceptance: 18-01-2012
Conflict of Interest: NIL
Source of Support: NONE

Introduction

Combretum roxburghii(roxburgh) belongs to a medicinally important family Combretaceae. Combretum and Terminalia genera of the family have been used in the treatment of syphilis, abdominal

pains, diarrhea and other ailments (Fyhrquist *et al.*, 2002). A number of species of Combretum(24) have been reported for antibacterial activity (Eloff, 1999; Martini *et al.,* 2004). Pharmacognostic features as cytotoxicity and antioxidant activity are the markers which point towards the effectiveness of plant material for the treatment of ailments like cancer, parasitic diseases etc. Plant kingdom is rich in wide variety of free radical scavenging molecules, such as phenolic compounds(e.g. phenolic acids, flavonoids, quinines, coumarins, lignin's, stilbenes, tannins), nitrogen containing compounds(alkaloids and amines),vitamins, terpenoids(including carotenoids) and some other endogenous metabolites, which are rich in antioxidant activity (Zheng and Wang., 2001 and Cai *et al.*, 2003). In general, antioxidant systems either prevent reactive species from being formed, or remove them before they can damage vital components of the cell. The reactive oxygen species are produced by our body's use of oxygen, such as in respiration and some cell-mediated immune functions and include hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), and free radicals such as the hydroxyl radical (·OH) and the superoxide anion (O_2^-). The hydroxyl radical is particularly unstable and will react rapidly and non-specifically with most biological molecules. Free radicals or reactive oxygen species(ROS) are also generated through environmental pollutants, cigarette smoke, automobile exhaust, radiation, air pollution, pesticides, etc. When the generation of these free radicals or ROS goes beyond the antioxidant capacity of a biological system, it gives rise to oxidative stress (Mikulikova & Popes., 2001). Oxidative stress plays a role in heart diseases, malaria, neurodegenerative diseases, cancer, AIDS and in the aging process (Sian *et al.,* 2003).

Epidemiological studies have shown that many of these antioxidant compounds possess anti-inflammatory, anti-atherosclerotic, anti-tumor, anti-mutagenic, anti-carcinogenic, antibacterial or antiviral activities to a greater or lesser extent(Owen *et al.*,2000; Sala *et al.*, 2002).Numerous types of bioactive compounds have been isolated from plant source of which some are currently in pre-clinical/clinical trials. Some species from the Combretaceae family have been reported to contain antioxidant activities. Masoko and Eloff, 2007 have reported antioxidant potential of 24 African combretum species, In previous work, Masoko *et al.*(2005) indicated that six selected Terminalia species possessed antioxidant activity. Amongst Combretum species, *Combretum woodie* has shown significant antioxidant and anti-inflammatory potential (Eloff *et al.*, 2005). Several compounds of interest such as flavonoids, stilbenes, cyclobutanes and triterpenoids have been isolated from *Combretum erythrophyllum* (Martini *et al.,* 2004). However, Indian species as *Combretum roxburghii* is yet to be investigated for pharmacological markers like antioxidant and cytotoxic potential to ascertain the significance of the plant for deriving useful compounds for clinical applications. This study aims to fulfill the gap.

Materials and methods

*Collection of plant material***:**

Leaves and bark of *Combretum roxburghii* was collected from medicinal germplasm garden of Regional Plant Research Centre, Bhubaneswar in the months of February, May, July and October.

Phytochemical analysis

Phytochemical analysis was conducted using standard protocols (Sofowora,1993 and Trease and Evans.,1997). A brief account of the different tests conducted was as follows:

Phlobotannin: Fresh leaf and bark powder of *Combretum roxburghii were* grounded with distilled water to make a solution. Then the mixture was filtered & the filtrate was taken as the sample. 1

ml of aqueous 1% HCl was added to 1 ml of sample followed by boiling. A red precipitate is indicative of presence of phlobotanins.

*Alkaloids***:** 1 ml of methanolic extract *was* filtered. Then 2 ml of 1% aqueous HCl was added to it. Thereafter it was heated for few minutes. 2 drops of dragondroff reagent was added to the solution. Reddish brown precipitate with turbidity depicts alkaloid's presence.

*Flavonoids***:** To 5 ml of methanolic extract, 1 ml of 10% NaOH solution was added. From the side of the beaker 2 drops of concentrated HCl was added. Yellow colour turning to colourless is an indication of presence of flavonoids.

*Anthraquinone***:** To 1 ml of methanolic extract, 2 ml of 5% KOH was added. Then the solution was filtered. Change in colour was observed. Pink colour shows the presence of anthraquinones.

*Saponins***:** About 2 ml of 1% sodium bicarbonate was added to 1 ml of methanolic bark extract and shaked. Lather like formation persistent for some time is indicative of presence of Saponins.

*Steroids***:** 100 µl methanolic extract of *Combretum roxburghii* leaf and bark was taken in a test tube and 400 µl of acetic anhydride was added to it. Then 1-2 drops of concentrated sulphuric acid was added to it. Brown ring at the boundary of mixture shows the presence of steroids. (N.B. Test tube was kept in ice as exo thermic reaction occurs.)

*Glycosides***:** 100 µl methanolic extract of *Combretum roxburghii* leaf and bark was taken in a test tube and 400 µl of acetic anhydride was added to it. Then 1-2 drops of concentrated sulphuric acid was added to it. Blue-Green colour shows the presence of glycosides.

Tannin: 1gm of sample added with 100ml of distilled water, boiled and cooled, and then filtered. 1% ferric chloride was added drop wise to the filtrate. Green black precipitate shows the presence of tannin.

Processing of Plant Material for solvent extraction:
In order to get the most active plant extract, sample was collected in four months namely February, May, July and October. Leaves and bark were washed thoroughly and dried at room temperature. Thereafter they were dried completely in oven at 50° C for one hour and ground to fine powder in a mechanical grinder of Lexus make. The materials were then packed in zipper polythene bags and stored at room temperature in dark until used.

Preparation of Extracts:
Leaf and bark powders were subjected to serial solvent extraction and five extracts hexane, DCM, chloroform, acetone, methanol were prepared respectively. These extracts were further concentrated by using Buchi (R-200) Rotavapour. The concentrated semi-solid extracts were stored in air tight screw cap vials and kept in refrigerator till further use (Mohapatra *et al.*, 2010).

Cytotoxic activity
Preliminary cytotoxic activity was conducted using brine shrimp assay(Meyer *et al.*,1982) Brine shrimp (*Artemia salina*) eggs were incubated for 48hrs (1.8gm of black salt in 100ml of distilled water) to get the desired growth of the larvae for biological evaluation. Stock solution of different extracts was prepared at a concentration of 20µg/ml. Extract was evaluated at the doses 25 and 50µg/ml. For each dose level three replicates were used. Motility, readings were taken every hour up to 4hours.
After 24hrs the final reading was taken and percentage of inhibition was calculated by comparing the treated samples with the controls. Standard deviation was also calculated. Active fraction of the

species was subjected to live cell assay and apoptosis tests.

Trypan blue dye exclusion method

Jurkat cells (tumor cell lines) were collected from ATCC (Global bioresource centre) were cultured in IMDM media with 10% FBS in presence of antibiotics, L-Glutamine and were maintained in a CO_2 incubator at 37degree Celsius with 5% CO_2 . Cells were plated in a 24well culture(Nunc) plates at 0.5 X 10^6 cells/ml. Fraction was diluted in media and were added at doses of 0,.01, 0.1, 1 and 10microgram/ml and were incubated for 24 hrs. Equivalent amount of media was added to the control wells. The efficacy of cell death was determined by trypan blue dye exclusion method. Cell viability data was plotted.

Apoptosis test

Test was conducted as per the protocol of Koopman *et al (*2004) and Vermes *et al* (1995). Jurkat and B16(both tumor cell lines) were plated as above and after 24 hrs were harvested and subjected to apoptosis study using apoptosis staining kit(BD Bioscience) as per manufacturer's protocol. Cells were washed twice with PBS and were suspended in binding buffer and transferred to FACS tubes. Cells were stained with Annexin V conjugated to PE and 7AAD. After 10 minutes of incubation at room temperature , 0.5ml of binding buffer was added to each tube. Samples were subjected to Flow analysis, data were acquired using FACS Caliber(BD bioscience) and was analysed using CellQuestPro software(BD Science).

TLC based qualitative analysis for Antioxidants

Qualitative screening of the constituents in each of the leaf and bark solvent extracts of *Combretum .roxburghii* for antioxidant activity was done by TLC analysis. About 5µl of each sample was loaded on the TLC sheet and the chromatograms were developed in following solvent systems:

i) Ethyl acetate/methanol/water (40:5.4:4) [EMW] (polar neutral);
ii) Chloroform/ethylacetate / formicacid (5:4:1) [CEF] (intermediate polarity/acidic);
iii) Benzene / ethanol / ammonium hydroxide (90:10:1) [BEA] (Nonpolar / basic) (Kotze and Eloff., 2002).

In order to detect antioxidant activity, qualitative 2,2 diphenyl-1-picrylhydrazyl(DPPH) assay was carried out.The plates were first air dried and then the chromatograms were sprayed with 0.2%,2,2,diphenyl-2-picryl-hydrazyl in methanol, as an indicator(Deby and Margotteaux,1970).The presence of antioxidant compounds were detected by yellow spots against a purple background.

Quantitative analysis using radical scavenging DPPH assay

Radical scavenging antioxidant activity was conducted by the standard protocol of Sanchez *et al* (1999). Ascorbic acid was used as standard antioxidant and IC50 of solvent extracts was compared with the standard.

Results and Discussions

Phytochemical analysis

Phytochemical tests of the plant *Combretum roxburghii leaf* and bark showed the presence of flavonoids, tannins and saponins. Out of these flavonoids is a class of compounds which are known for their medicinal potential. In general they have been reported for their antioxidant, anti-estrogenic and anti-proliferative properties(Gretchen *et al*, 2008: Mcgaw *et al*, 2001). Detection of flavonoids in the plant species was expected as in another species of the same genera namely *Combretum erythrophyllum,* flavonoids have been isolated as active principle (Martini *et al*, 2004). Similarly flavonoids have been reported from *Terminalia alata* (Srivastava *et al*, 1999). The most common bioactive flavonoids Catechin has been reported from a

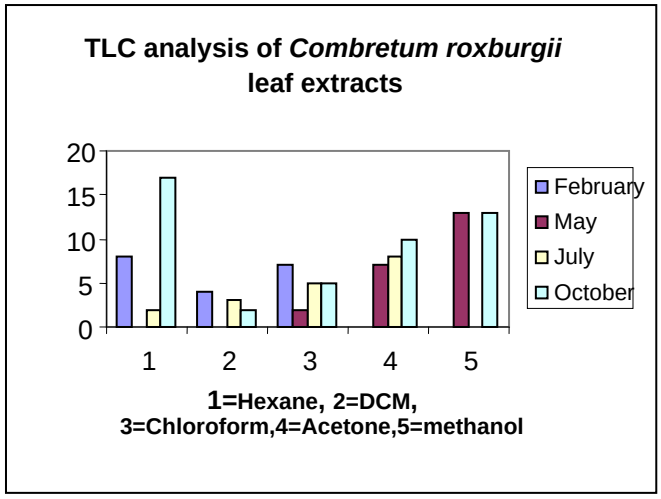
number of medicinal plants *Saraca asoka* and *Combretum albiflorum* (Vandeputte *et al*, 2009). Thus, presence of flavonoids in the species indicates towards the medicinal potential of the plant.

Antioxidant activity

As shown in Figure 1, it can be visualized that a large number of antioxidant bands are present in most of the extracts suggesting that *Combretum roxburghii* is positive for antioxidant activity. However, a seasonal variation is evident from the above as hexane, DCM and chloroform extracts of leaf showed maximum antioxidant bands(19each) in the month of February and October where as in other two extracts antioxidant bands were absent in the month of February but present throughout the year from May to October(31). Thus, it can be derived that in non-polar extracts antioxidant molecules are rich in the month of February and October, whereas polar extracts consists of molecules in all the other month. Thus, no particular season in case of leaf collection can be pointed out as an ideal season for antioxidant activity. As shown in Figure 2, in case of bark extracts once again the first three extracts were devoid of any antioxidant bands in the month of may and July, where as polar extracts namely acetone and methanol extract of bark were rich in antioxidant molecules from may to October. Results are in confirmation with an earlier study in which 24 species of Combretum genus were explore for antioxidant bands using the similar antioxidant assay. Maximum number of antioxidant bands were reported from *Combretum hereroense* (Masoko and Eloff, 2007) followed by 10 bands in *C. collinum*. In case of *Combretum roxburghii* in all the three solvents 27 antioxidant bands were found suggesting it to be richer as compared to its foreign counterparts.

Quantitative analysis of antioxidant activity is shown in Figure 3. IC50 of ascorbic acid was similar to that of acetone extract (between 39-9.5microgram). Thus, acetone extract could only be considered to be of significant antioxidant potential. Remaining extracts showed very little antioxidant activity as compared to the standard as well as acetone extract. Similar promising antioxidant activity was also found in the acetone extract of *Tilia argentia (*Demiray *et al*, 2009). In another study, aqueous extract of ripe fruits of *Terminalia chebula* have shown good antioxidant activity(Mahesh *et al*, 2007). Besides the members of Combretaceae family, other species have also been reported for their antioxidant potential some of these are *Ludwigia octovalvis* belonging to family Ongraceae(Shyur *et al*, 2005), *Solanum pennellii* of Solanaceae family(Frary et al, 2010)etc. Thus plant kingdom is a rich source of antioxidant molecules. This study has revealed the antioxidant potential of one such member.

Figure 1



Cytotoxic and apoptosis activity

Preliminary cytotoxic activity of all the five solvent extracts was tested in brine shrimp assay, which is a well known model for testing the cytotoxic activity and many novel compounds for cancer like piperydinyl-DES and pyrollidinyl-1DES have been evaluated by using the same test model (Badisa *et al*, 2009). In order to get the most active extract, plant material was collected in different time periods, All leaf extracts showed good activity in the months of July and October ranging from 56-100% and 86-100%respectively. Only hexane extract showed good

activity to the tune of 98% at a higher dose of 50microgram/ml in the month of February and 100 percent in the month of October and as per table 1, even antioxidant bands were maximum in these months suggesting that oil content/non-polar contents if need be isolated, then leaves of *Combretum roxburghii* should be collected in February or October. Acetone extract of leaf(Table 1) was found to be consistently active(100%)in cytotoxic activity as well as antioxidant activity from the samples collected in the July and October seasons. Further fractionation of the same was conducted to locate the active principle as in protocol (Fig.1). One pure active fraction was obtained which was further subjected to Trypan blue dye exclusion method, fraction showed a dose dependent cytotoxic activity(Figure 2). On confirmation of cytotoxic potential of the pure fraction, same was subjected to apoptosis test using Jurkat cells and B16(Human tumor cell lines). In apoptosis phosphatidyl serine(PS) which is normally find inside the plasma membrane is found outside the cells due to loss of phospholipids symmetry of the cell membrane. Annexin V binds to this PS in apoptosis, in case of the fraction tested this binding was not observed from which it can be concluded that cell death in case of the sample was due to some other mechanism but not due to apoptosis (figure 4) A large number of antioxidant molecules with cytotoxic principles are useful in combating a number of parasitic infections, keeping this in view pure fraction could be useful in other purpose like anti-malarial, anti-leishmanial and other parasitic infections (Ademola and Eloff, 2010). Thus, this study has provided lead which can be further utilized for evaluating other test models for different diseases.

Acknowledgements

Acknowledgements are due to NMPB, New Delhi for providing funding for the project fellows Sunita Sahoo and Dipti Ranjan Behra. Acknowledgements are also due to the Taxonomic team of the Institute.

Figure 2

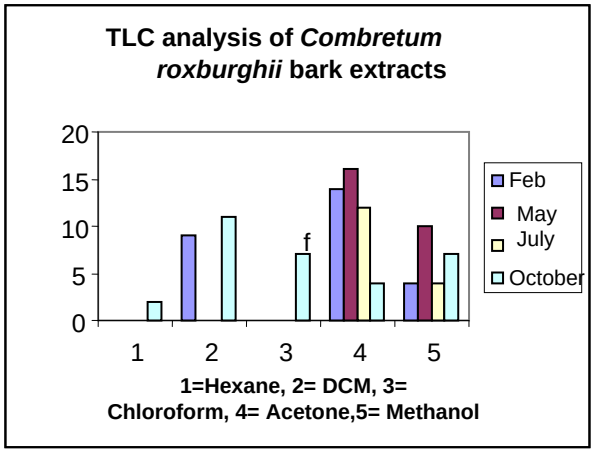


Figure 3

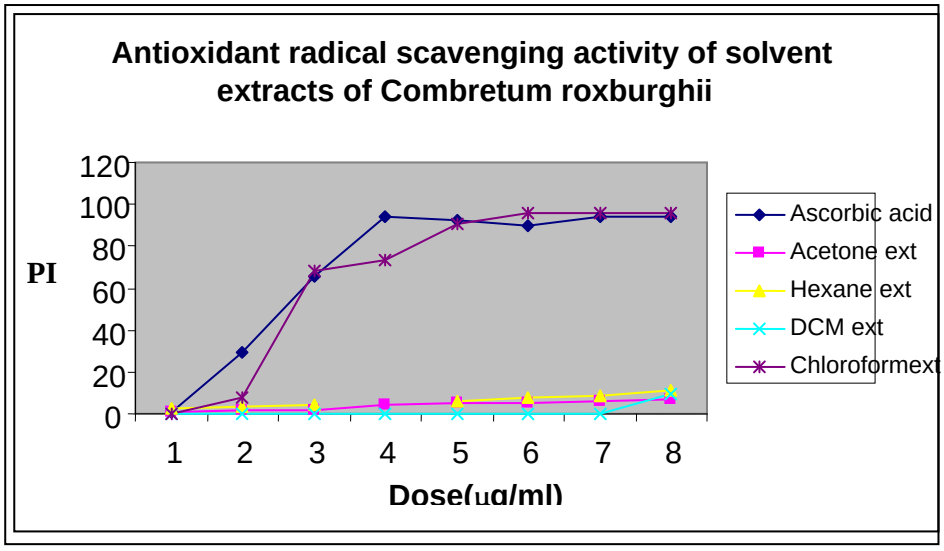


Table 1: Brine shrimp assay of Combretum roxburghii leaf extracts.

Extract	Dose (µg/ml)	Month of collection			
		Feb.	May	July	October
		%inhibition Mean±S.D	%inhibition Mean/S.D	%inhibition Mean±S.D	%inhibition Mean±S.D
Hexane	25	66.7±10.0	43.3 ± 2.9	56.7± 3.5	100.0 ± 0.0
	50	98.3 ± 2.9	45.0 ± 5.0	90.0 ± 5.0	100.0 ± 0.0
DCM	25	26.5 ± 4.2	26.7 ± 5.8	90.0 ± 8.7	95.3. ± 4.0
	50	56.9 ± 3.4	83.3 ± 2.9	90.0 ± 8.7	86.0.8 ± 0.0
Chloroform	25	36.7 ± 4.7	23.3 ± 7.6	95.0 ± 8.7	95.3 ± 5.0
	50	75.6 ±10.2	93.3 ± 2.9	86.7 ± 5.8	96 .6 ± 4.7
Acetone	25	13.3 ± 0.0	67.0 ± 2.9	100.0 ± 0.0	93.3.± 6.5
	50	37.8 ± 3.8	80.0 ± 13.2	91.2 ± 2.9	100.0 ± 0.0
Methanol	25	10.0 ± 5.0	58.0 ± 5.8	76.7 ± 2.9	89.0 ± 10.0
	50	31.7 ± 7.6	63.3 ± 7.6	81.7 ± 4.2	95.3 ± 8.1

Table 2: Brine shrimp assay of *Combretum roxburghii* bark extracts

Extract	Dose ((µg/ml))	Month of collection			
		Feb.	May	July	October
		%inhibition Mean±S.D	%inhibition Mean±S.D	%inhibition Mean±S.D	%inhibition Mean±S.D
Hexane	25	67.5±3.53	43.3± 6.3	52.0 ± 7.63	23.8± 8.0
	50	98.33±2.88	75.0± 13.2	100.0 ± 0.0	92.7± 12.7
DCM	25	25.63±4.43	70.7 ± 13.1	100.0 ± 0.0	91.0± 9.0
	50	35.89±8.88	95.0± 0.0	80.7 ± 18.1	81.7± 12.7
Chloroform	25	27.44±8.9	52.0 ± 6.9	95.0 ± 0.0	63.8 ± 8.5
	50	61.76±12.47	84.0 ± 7.6	95.3 ± 4.1	85.3 ± 4.7
Acetone	25	64.7±5.88	77.0 ± 6.1	46.1 ± 3.5	9.6 ± 0.1
	50	88.23±5.88	100.0 ± 0.0	100 ± 0	40.6 ± 3.5
Methanol	25	8.88±3.8	100.0 ± .0.0	90.0 ± 0.0	97.0 ± 5.2
	50	40.11±6.8	95.0 ± 3.53	95.3 ± 4.0	100± 0

Figure 5: Flow chart of Isolation of active principle from *Combretum roxburghi*

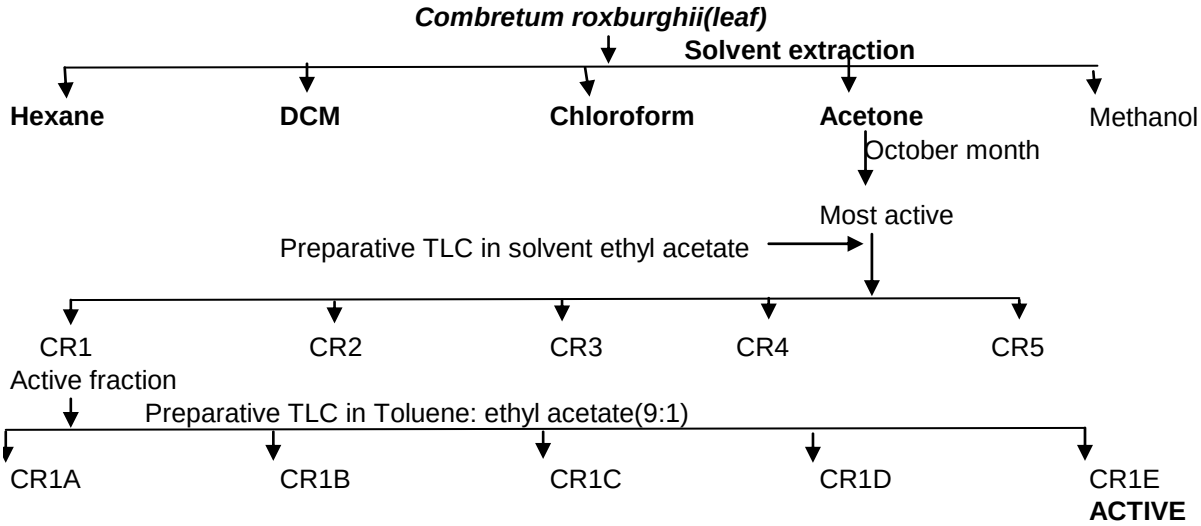
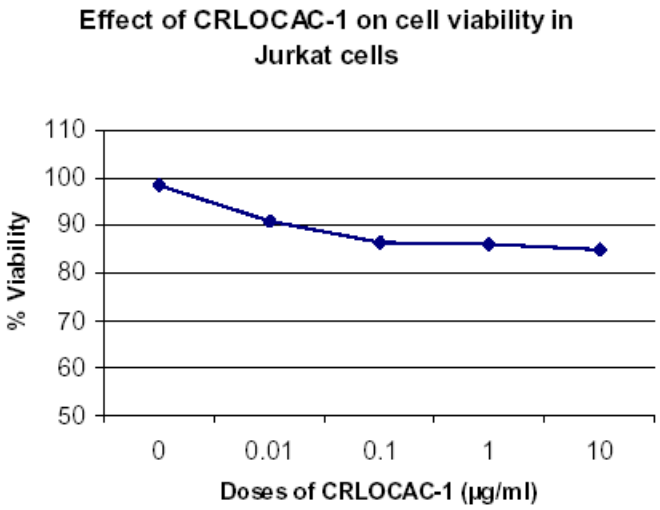


Figure 6



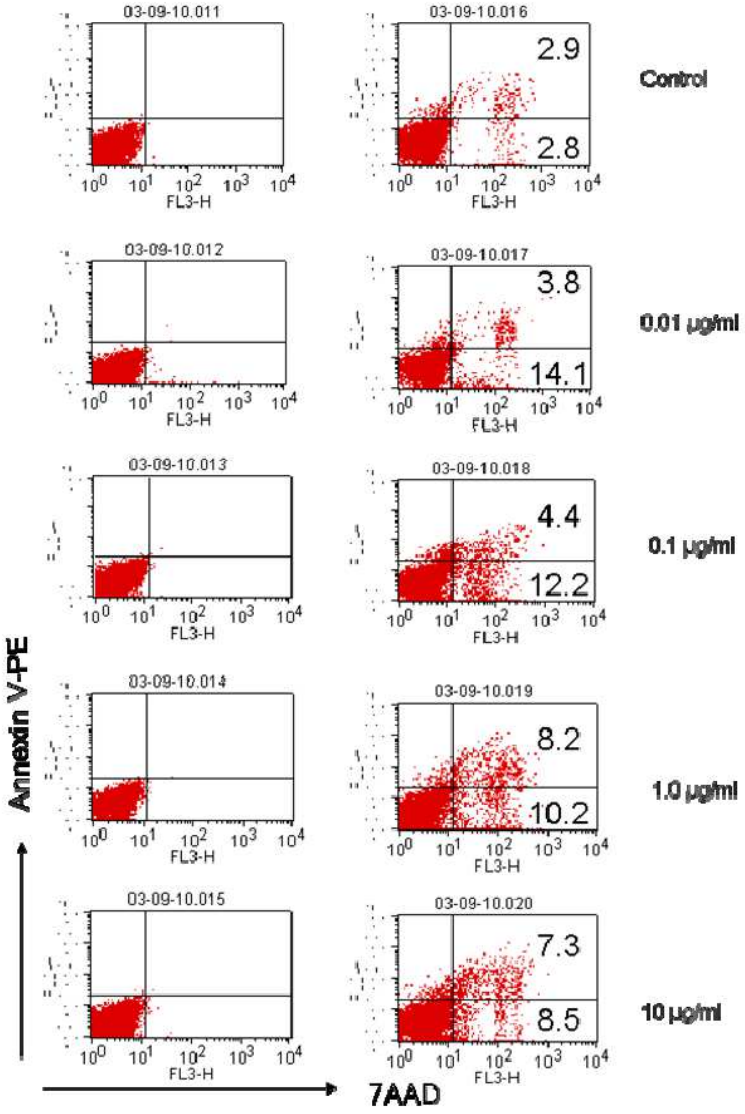


Figure 4. Study of apoptosis in Jurkat cells using varying doses of CRLOCAC-1

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