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RESEARCH ARTICLE

ANTIOXIDANT AND CYTOTOXIC ACTIVITY OF *CommiphoraMukul*(GUGGUL) EXTRACTS AGAINST *HeLa* CELL-LINE.

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Abstract

Commiphoramukul found in India, Pakistan and Bangladesh. Guggul gum resin has been used to treat various diseases including cancer, atherosclerosis, rheumatism and obesity. The crude extracts obtained from the resin of *Commiphoramukul* in different solvents like ethyl acetate, methanol, acetone, and hexane were analyzed for phytochemical study. Secondary metabolites steroids, quinines, gum and mucilage, fixed oil and fats were found in all the four different extracts of *Commiphoramukul* however, oxalate and alkaloids were not found in any extract. Ethyl acetate, methanol, acetone, and hexane extracts from method-1(soxhlet) and method2 (cold) were tested for their ability to scavenge DPPH radical. Ethyl acetate extracts exhibited higher antioxidant activity (45.56%) compared to other solvent extracts. Various concentration of *Commiphoramukul* plant resin extract were used for MTT assay and highest % inhibition/Cytotoxicity (75.68 %) was found by methanol soxhlet extract having lowest IC_{50} value (58.5µg/ml).

Introduction:-

India is a vast country where wide variations in soil, climate, latitude and altitude are available. Guggul (*Commiphoramukul*) is one of the most ancient medicines described in Ayurveda. Guggul with an aromatic odor, is the best medicine because it develops through the rays of hot sun on specific circumstances. *Commiphoramukul* (Arnott) Bhandari of Burseraceae family is highly branched, slow growing, , shrubby plant that grows wild in the arid rocky tracts of Rajasthan, Gujarat, Madhya Pradesh and Karnataka states of India, and in the Sind and Baluchistan states of Pakistan. Oleogum resin is the economically viable part of the plant. It is excreted by specialized ducts in plants, especially from stem-bark. As per constituents, it has 6.9% moisture, 0.6% volatile oil, resin 61%, gum 29.6%, and insoluble substances 3.2%.The oleo-gum resin mixture of gum, mucilage, fixed oil, fats, terpenes, steroids, flavanones, and quinines. In 1986, Guggul was granted approval for marketing as a lipid lowering drug (Indian Pharmacopeia 2007; 2038-2040). Several products of standardized formulations of *Commiphoramukul* are already in human use as cholesterol lowering agent.

The present study of qualitative analysis was carried out for various phytoconstituents. Solvents like methanol, acetone, hexan, and ethyl acetate were taken to prepare the resin extracts in order to distinguish the presence of the active components. The Phytochemical screening demonstrated the vicinity of all the concoction constituents like steroids, phenolic, flavonoids, quinines, and the conspicuous absence of oxalate, tannins, and alkaloids. Experiments to test antioxidant activity of ethyl acetate crude extracts from *Commiphoramukul* (guggul) were also carried out. Oxidative stress seems to play a significant role in many human diseases, including cancers and studies have shown that antioxidant rich diet can prevent onset of these diseases. We investigated the inhibitory effect of guggul extracts on growth of the human cervical cancer(*HeLa* cell line).The current study deal with evaluation of gum-resin part of

the guggul for possible cytotoxicity followed by MTT assay for methanol(cold) and methanol (soxhlet/hot) and ethyl acetate (cold) and ethyl acetate (soxhlet/hot) crude extract.

Materials and methods:-

Plant Identification:-

Plant samples were collected from the Balamar Forest-Ambaji Arrival Mountains, Gujarat, (India) in the end of month of December 2014.The plant was identified and authenticated by Dr. Dharmik V. Barot in Gujarat Agriculture University, India.

Collection and preparation of plant materials:-

The resin of *Commiphoramukul* was collected, chopped in small pieces and dried in the shade. The dried resin was stored at room temperature (30°c±2) until use. Purity of resin was tested by taking 2.5g of the dried resin in 25ml 95% methanol followed by boiling for 20minutes. Formation of precipitate in filtrate after addition of 2.5mL distilled water confirmed presence of resin.

Preparation of extracts:-

Hot extraction:-

Plant materials were extracted (Murugan and Saranraj, 2011) sequentially with Hexane (69°C), ethyl acetate (77°C), acetone (56°C), methanol (64°C) in order of increasing polarity. Fifteen grams (15g) of the plant resin was soaked in 100 ml of the extraction solvent and extraction was carried out in soxhlet apparatus until they became colorless. The extract obtained from the method were kept in the Petri plate for 48hrs at room temperature (30°c±2) to evaporate the solvent. The dried extract was kept in a screw cap tube. All the extracts were kept in fridge (7-8°C) until use.

Cold extraction:-

Plant materials were extracted (Rajaselvam*et. al.*, 2012) sequentially with Hexane (69°C), ethyl acetate (77°C), acetone (56°C), methanol (64°C) in order of increasing polarity. Fifteen grams (15g) of the plant resin was soaked in 100 ml of the extraction solvent and extraction was carried out at room temperature (30°c±2) for 92 hr on rotary shaker 150 rpm. Residual of plant materials was filtered through Whatman® filter paper-1. Extraction per solvent was carried out once. The extract were filtered and kept in the Petri plate for 48hr at room temperature (30°c±2) to evaporate the solvent. The dried extract was kept in a screw cap tube. All the extracts were kept in fridge (7-8°C) until use.

Extraction Efficiency:-

Extraction efficiency was calculated for each solvent obtained by hot and cold extraction.

%Yield efficiency= [Weight of extracted sample/Weight of sample] ×100

Phytochemical test:-

Qualitative Phytochemical analysis:-

The presence of alkaloids, flavonoids, glycosides, reducing sugars, saponins, tannins and terpenoids were tested qualitatively using the standard procedures to identify the constituents. Test for steroids:Test for steroids was performed by the method given by Siddique *et. al*, (2007), Edeoga*et. al*, (2005). Test for phenols: Presence of phenolic compounds were determined by $FeCl_3$ test described by SaxenaK.andSaxena J. (2012). Test for Flavonoids: Test for flavonoids were performed by the method given by Siddique *et. al.* (1997). Test for saponins: Test for saponins was carried out by method described by Parekh and Chanda, (2007). Test for terpenoids: Test for terpenoids were performed by the method given by Siddique *et. al*., (2007) Edeoga*et. al*, (2005). Test for tannins: Tannin was determined by the method given by Saxena K. and Saxena J, (2012). Test for quinines: 1mLof extracted was treated with conc. HCL and observed for the formation of yellow precipitate or (colouration).Test for cardiac glycosides: Test for glycosides was performed by the method given by Parekh and Chanda (2007). Test for Phlobatannins: (Precipitate test): Deposition of a red precipitate when 1ml of extract was boiled with 0.5ml of 1% aqueous hydrochloric acid was taken as evidence for the presence of Phlobatannins. Test for oxalate: A few drops of ethanoic acid glacial to 1ml portion of extract was added. A greenish black colouration indicates presence of oxalates. Test for Gum and Mucilage:About 10ml of the extract was slowly added to 25ml of absolute alcohol under constant stirring. Precipitation indicates the presence of gum and mucilage. Test for Fixed oils and Fats:A drop of concentrated extract was pressed in between two filter papers and kept undisturbed. Oil stain on the paper indicates the presence of oils and fats. Test for alkaloids: Test for alkaloids were performed described by Raffauf R.F. (1962).

Antioxidant Bioassay (DPPH Radical Scavanging activity):-
The inhibition effect of eight different extracts (ethyl acetate, acetone, methanol and hexane extracts from both hot and cold extractions)on free radical DPPH was studied using the DPPH radical-scavenging method as described by Goz*et. al* (2009). One milliliter (1 ml) of different concentration (from 0.1, to 1.0 mg/ml) of the plant extracts in methanol were mixed 1 ml of 0.1 mM DPPH solution, shaken vigorously and allowed to stand for 40 minutes in dark condition before measuring the absorbance with a UV spectrophotometer at 517 nm. A sample of the dissolving solvent (methanol) with no plant extract was used as a negative control. Ascorbic acid was used as a positive control and also used as a standard. The inhibition effects of the extract on free radical DPPH were expressed as follows:

% inhibition= [(Ab of control- Ab of sample)/Ab of control] × 100
*Where Ab is Absorbance

Method for cell line growth:-

HeLa cervical cancer cell line was procured from National Center for Cell Science, Pune (NCSS). Assay was performed at the Department of Zoology, Gujarat University. Cells were grown in DMEM medium (Hi-Media), supplementedwith 10% fetal Bovine Serum (FBS) in a humidified 5% CO2 incubator at 37ºC.When cells reached 90% confluence, they were trypsinised and plated after counting. The media was discarded and the cell monolayer was washed with 2-4 ml of trypsine- EDTA solution. Detached cells solution was centrifuged at 1200 rpm at 24ºC for 5 minutes to obtain a cell pellet. The pellet was resuspended in 10 ml of fresh media supplemented with 10% FBS.One fifth of the resuspended cells (2ml) of solution were transferred back into the culture flask to which 8mlof supplemented media was added for sub culturing into 50ml flask. The cells were incubated at 37ºC in the 5% incubator. The rest of the cells were seeded into plates and used for the experiments.

***In Vitro* Assay:-**
Cells were resuspended in eppendorff tube in 1 part of 0.4 % trypan blue (100µl) and 1 part of cell suspension (100µl) and incubated for 3 minutes at room temperature (36ºC± 2) and counted on heamocytometer. If the cell number was higher than 25 cells in the 25squares, the cell suspension was diluted with 4-(2-hydroxyethyl)-piperazine-1ethanesulfonic acid-buffered saline solution (HEPES-BSS) and cells were recounted. The Use the formula above to determine the number of cells per milliliter concentration.

Number of cells/ ml of cells suspension= number of cell counted ×106 / 25 squares

Cell plating-:
Concentration obtained from the above step, the cell was diluted to the desired concentration with supplemented media. Cells were seeded in 96- well plates at 1×10^6 cells /ml for the MTT assay. In the assay was carried out take a 100µl of the cell suspension was delivered into each well using micro-pipette. Plate were incubated for 24 hours at 37ºC in a 5% CO_2 incubator or until they reached ~90% confluences before the treatment with plant extracts.

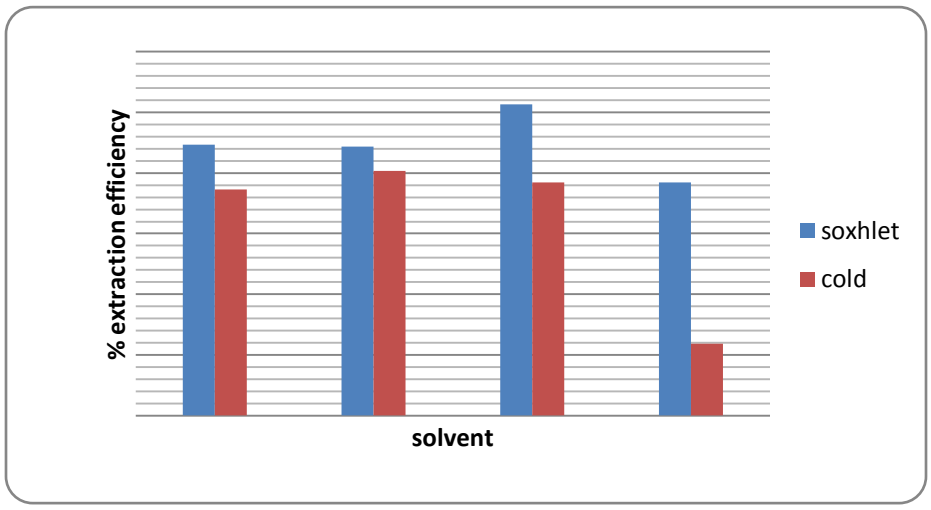
Four plant extracts from Commiphoramukul for testing Cytotoxicity:-
Methanol extractsby Hot and cold method and Ethyl acetate extractsby hot and cold method weredissolved in DMEM medium to make a stock solution of 1mg/ ml and add 1ml of 10% DMSO solution. The Following concentration of extracts were taken for dose, 500µg, 250µg, 125µg, 62.5µg, 31.5µg. Experiment was performed in tri-plicate. Plate were incubated at 5% CO2, 37ºC for 24 hours. The above listed concentration of extracts was used for cytotoxicity (MTT) assay.

***In vitro*Cytotoxicity of Resin crude extracts:-**
Preparation of resin crud extracts (1mg/ml stock solution in media).Plating of cell in 96 ELISA plate (10000cells/well) 24 hour acclimatization (cells are allowed to grow under incubate conditions usually 37ºC with supplemental 5% CO2). Addition of resin crude extract in decreasing concentration 500 µg/ml to 31.5µg/ml. Incubate with resin crude extract for 24 hour. Replace media and add 10µl of MTT reagent and Incubate for 4 hour followed by monitoring using inverted microscope andadd 100µl solubilizing reagent (0.004%HCL in isopropanol) to solubilize the formazoncrystals. Take OD at 630 nm % Survival rate was calculated for the extract dose given to them by the following formula, % Survival rate= [(Sample-Blank)/ Control-Blank] ×100 Calculate IC_{50}Distribution map 96 well plate.

Results and discussion:-

Table 1:- Showing the extraction efficiency from crude samples.

Method	Ethylacetate	Methanol	Acetone	Hexane
Hot extraction(Soxhlet)	44.56%	44.24%	51.28%	38.41%
Cold extraction	37.24%	40.25%	40.25%	11.8%



Graph 1: Extraction efficiency of Soxhlet and Cold method for different solvent.

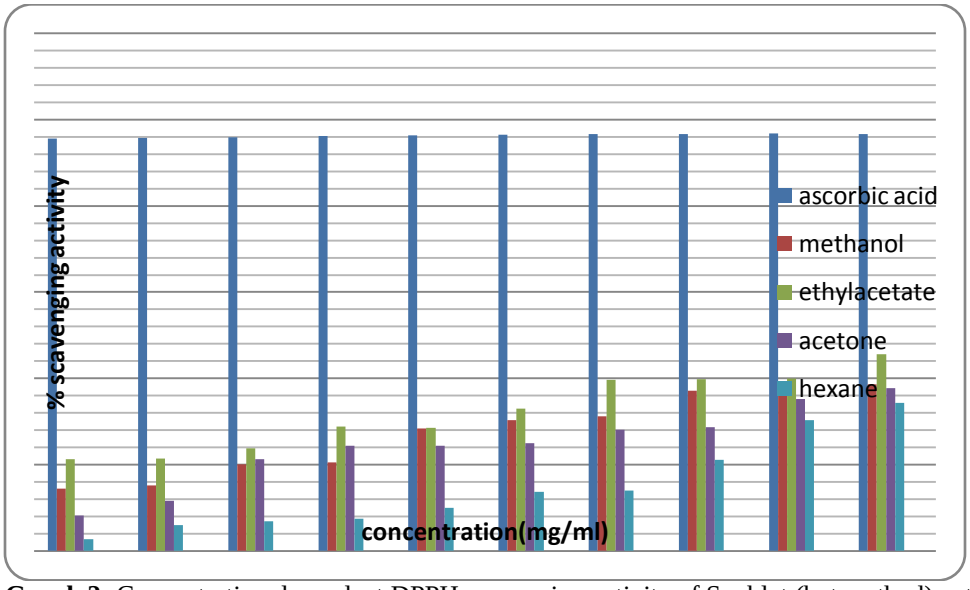
Phytochemical analysis of medicinal plant extract:Qualitative Phytochemical Screening of Soxhlet and Cold extract of resin from CommiphoramukulWhere, S=Soxhlet extracts, C=Cold extracts.

Phytochemical test	**S. Ethyl acetate**	**C. Ethyl acetate**	**S. methanol**	**C. methanol**	**S. acetone**	**C. acetone**	**S. hexane**	**C. hexane**
Steroids (Lieberman-Burchadt test)	+	+	+	+	+	+	+	+
Phenoles (Ferric chloride test)	+	+	+	+	+	+	-	-
Flavonoides (Alkaline reagen test)	+	+	-	-	-	+	+	+
Saponines(Foam test)	+	+	-	-	-	-	+	+
Terpenoids(Salkowski's test)	+	+	-	-	+	+	+	+
Tannins	-	-	+	+	+	+	-	-
Quinines	+	+	+	+	+	+	+	+
Cardiac glycosides (Kellel-killan's test)	+	+	-	-	-	-	+	+
Phlobatannins (Precipitate test)	-	-	+	+	+	+	-	-
Oxalate	-	-	-	-	-	-	-	-
Fixed oil and fats	+	+	+	+	+	+	+	+
Gum and mucilage	+	+	+	+	+	+	+	+
Alkaloids	-	-	-	-	-	-	-	-

Result for Antioxidant activity:- DPPH assay

Table 1:- concentration dependent DPPH scavenging activity of Soxhlet various extract.

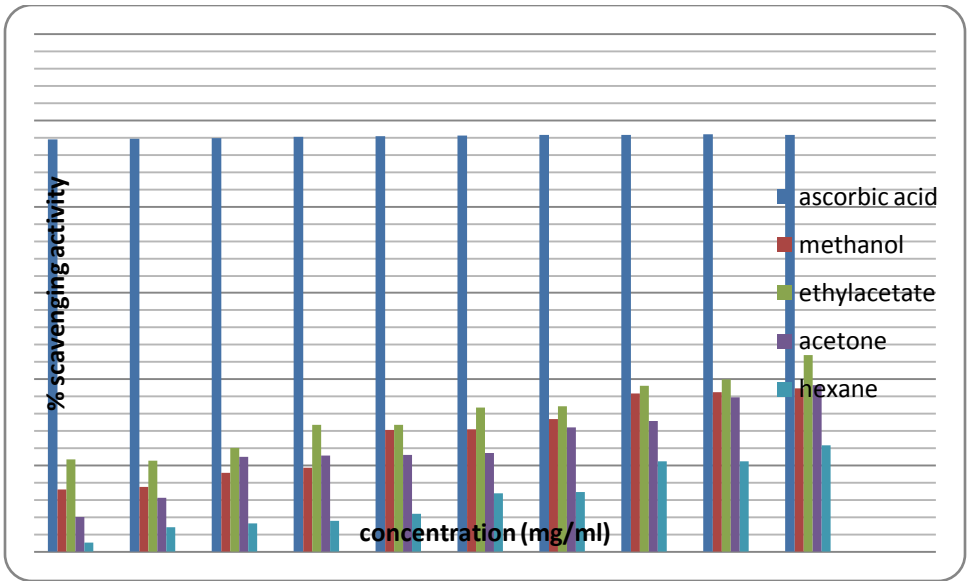
Concentration(µg/ml)		%scavenging activity			
	Ascorbic acid	Ethyl acetate	Methanol	Acetone	Hexane
0.1	95.66	21.25	14.48	8.2	2.6
0.2	95.76	21.44	15.12	11.22	5.9
0.3	95.95	23.75	20.1	21.22	6.9
0.4	96.24	28.74	20.53	24.41	7.4
0.5	96.43	28.48	28.41	24.31	9.9
0.6	96.53	32.99	30.33	24.88	13.72
0.7	96.62	39.64	31.25	28.07	14.02
0.8	96.72	39.83	37.12	28.63	21.12
0.9	96.82	40.01	38.13	35.21	30.37
1.0	97.71	45.56	38.58	37.65	34.37



Graph 2: Concentration dependent DPPH scavenging activity of Soxhlet (hot method) extracts.

Table 2:- concentration dependent DPPH scavenging activity of Cold various extract.

Concentration(µg/ml)		% scavenging activity			
	Ascorbic acid	Ethyl acetate	methanol	Acetone	Hexane
0.1	95.66	21.43	14.41	8.1	2.1
0.2	95.76	21.06	14.96	12.46	5.75
0.3	95.95	24.01	18.27	21.95	6.63
0.4	96.24	29.34	19.46	22.32	7.1
0.5	96.43	29.43	28.19	22.43	8.8
0.6	96.53	33.48	28.37	22.88	13.46
0.7	96.62	33.76	30.77	28.74	13.75
0.8	96.72	38.45	36.68	30.32	20.87
0.9	96.82	39.92	37.00	35.81	20.97
1.0	97.71	45.53	37.92	38.60	24.68



Graph 3: Concentration dependent DPPH scavenging activity of Cold extracts.

Result of In vitro screening:-

Revival of cells:-

Cell count of the revival of cells: Live/Dead (L/D)By using Haemocytometer for the cell counting live cell was found 122 and dead cell was found th18 therefore total number of cell count was 140.Viability was determine by

Formula =Live cells /Total cells×100
=122/140×100
=87.14%

Flask containing more than 87.14% viable cell were preceded further for phytochemical screening.

MTT Assay:-

Cells in each well were visually confirmed for the formation of formazon crystals prior to addition of solubilizing agent.Result of MTT assay obtained as % inhibition at various concentration of plant extract when plotted after log transformation of concentration we found increased % inhibition with increasing concentration of resin plant extract. IC_{50} values were extrapolated from the semi log graph as shown in Table-3.Where Ethyl acetate cold extract shown highest % inhibition as compared to all methods.

Table3:- Result of MTT assay using various plant resin extract on HeLa cell line suspended in DMEM medium with ethyl acetate, methanol. Where, %= % growth inhibition.

Concentration (µg/ml)	Ethyl acetate(Cold)	Ethyl acetate(Soxhlet)	Methanol (Cold)	Methanol (Soxhlet)
500	56.59%	61.38%	65.43%	75.68%
250	40.92%	53.84%	65.16%	57.37%
125	34.50%	46.53%	53.17%	54.92%
62.5	21.12%	39.15%	41.06%	51.10%
31.25	16.95%	36.06%	40.91%	47.11%

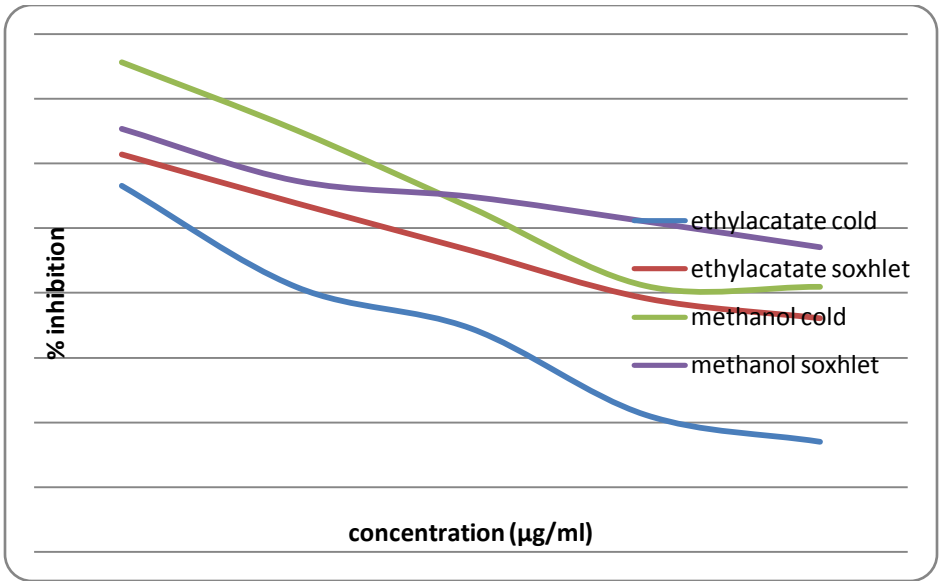


Figure 6:- various concentration of *Commiphoramukul* plant resin extract using for MTT assay and % inhibition (cytotoxicity).

IC_{50} values were extrapolated from the semi log graph as shown in Table-3.Where Ethyl acetate cold extract shown maximum % inhibition as compared to other methods.

Table3:- Inhibition concentration of Commiphoramukul plant resin extract.

Ic50	Concentration(µg/ml)
Ethyl acetate (cold)	390µg/ml
Ethyl acetate (soxhlet)	185µg/ml
Methanol(cold)	105µg/ml
Methanol(soxhlet)	58.5µg/ml

Discussion:-

Recently safety considerations, public's perception and risk reduction of chronic diseases by consumption of fruits and vegetables, have geared interest in the search for natural antioxidants (Dastmalchi*et al*., 2007). Excess generation of free radicals cause depletion of immune system antioxidants, alter in gene expression and induce abnormal proteins and contribute to more than one hundred disorders in humans including atherosclerosis, arthritis, ischemia and reperfusion injury of many tissues, central nervous system injury, gastritis, cancer and AIDS. Antioxidant can protect the body by preventing the formation of free radicals, by bringing interruption in Free radicals attack, by scavenging the reactive metabolites or by converting them to less reactive molecules (Hegde and Joshi, 2009).

Different solvent system used in this study revealed soxhlet extraction using acetone as best extraction solvent for resins but best cytotoxicity was shown by methanol extracts and best antioxidant activity was shown by ethyl acetate extracts, these results may indicate different active component in each solvent system as also reported by Musharaff*et. al.*(2011).The proton-donating ability of *Commiphoramukul* extract was evaluated through DPPH assay. IC_{50} value of Guggulsteronwas found to half (58.5µg / ml)of standard ascorbic acid in DPPH assay, similar results (IC_{50} = 46.87 µg / ml) was obtained in another study (Karan *et. al*.2009) indicating need of further research onscavenging free radicals.

Reducing power of any extract will be given by the amount of reductones present in them. The ability of the hydroxyl groups present in the flavonoids / phenolics to reduce the free radicals by donating their electrons will determine their activity. Dose dependent reducing ability of any extract exerted antioxidant action by breaking the free radical chain by donating hydrogen atom (Duh *et al.*, 1999). Cytotoxicity of all four extracts were measured by MTT assay. Methanolic extract of *Commiphoramukul*shown highest cytotoxicity (78.56%) suggesting the presence

of secondary metabolite which can be evaluated for development of anticancer agent. So, this antioxidant potentiality of Guggulsteron obtained from *Commiforamukul*is an important approach for the management of oxidative stress ailments.

Conclusion:-

This work has demonstrated that the ethyl acetate extracts of *Commiforamukul* possesses promising antioxidant activity and methanolic extracts shown cytotoxic potentiality, thereby lends support to the traditional use of the plant in infectious and inflammatory disorders. However, further studies are needed to be conducted to understand the exact mechanisms of such actions and to isolate the active principles responsible for the observed activity.

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