World Journal of Pharmaceutical and Life Sciences
WJPLS
www.wjpls.org
SJIF Impact Factor: 4.223

IN VITRO STUDIES ON THE EFFECT OF GARDENIA GUMMIFERA METHANOL EXTRACTS IN MDA-MB-231CELL LINES

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Article Received on 08/08/2017 **Article Revised on 29/08/2017** **Article Accepted on 20/09/2017**

ABSTRACT

The leaves of *Gardenia gummifera* is commonly known as gummy gardenia/cambi gum tree. It is traditional medicinal plant grown in India, and also has many properties like include anthelmintic, antispasmodic, carminative, diaphoretic, expectorant and used for the treatment of dyspepsia, flatulence for cleaning foul ulcers and wounds. These leaves extracts are found to have antioxidant and anticancer activities. Cycloartanes is the active principle which is responsible for anticancer beneficial effects. This work was aimed to study the effect of *Gardenia gummifera* on MDA-MB-231 cell lines and the Methanolic extracts of *Gardenia gummifera* leaves has found to have phytochemical compounds that are moderate inhibitory scavenging and more anticancer activities.

KEYWORDS: *Gardenia gummifera*, Cycloartanes, MDA – MB – 231 cell lines.

INTRODUCTION

The history of Ayurveda goes back to 5000 BC. It was developed through daily life experiences with the mutual relationship between mankind and nature. It has 2000 plant species for their therapeutic potentials. Historically, plants have provided a source of inspiration for novel drug Compounds, as plant-derived medicines have made large contributions to human health and well-being. In our country since from Vedic period, we are using crude plants as medicine. Presently Indian health care possess both traditional and modern systems of medicines, like Ayurveda, Siddha, Unani and unorganized systems like folk medicine.[1]

Breast cancer is the common cause of cancer death in women. Globally, every year more than one million women are diagnosed with breast cancer and more than 4, 00,000 are likely to die from this disease. Present therapies include surgery, radiation and chemotherapeutic agents. There are limitations with these and side effects are common. Currently gemcitabine is used for breast cancer. However gemcitabine treatment results only marginal survival advantage and is associated with many side effects including development of drug resistance. Thus there is need for the development of new therapeutic agents and new compounds isolated from plants with the existing anticancer agents that should show better outcome.[2]

MDA-MB-231 CELL LINE

Organism	**:**	*Homo sapiens*, human
Tissue	**:**	Mammary gland/breast
Disease	**:**	Adenocarcinoma
Age	**:**	51 years adult
Gender	**:**	Female
Morphology	**:**	Epithelial
Growth Properties	**:**	Adherent

The MDA – MB – 231 cells in the control culture samples were homogenously distributed into the culture field. They exhibited polygonal shapes with distinct boundaries and homogenous or slightly granulated cellular contents. The cells were thin and elongated with tapered ends in cytoplasmic branches (metastatic branches).

The MDA – MB – 231 cell line is an estrogen receptor alpha (ER)-negative human breast cancer cell line. It was derived from a metastatic adenocarcinoma of the mammary gland of a 51-year-old Caucasian woman, according to the data sheet of the American Type Culture Collection (ATCC). This adherent epithelial cell line that likely contains more than one cell populations is a highly aggressive, invasive and poorly-differentiated human breast cancer cell line. The MDA-MB – 231 cells display the invasiveness by mediating the proteolytic degradation of the extracellular matrix (ECM), including basement membrane and several mechanical barriers to the ECM, through the increased expression of matrix

metalloproteinases (MMPs), including gelatinases, en route to their destinations.[3]

Type IV collagen, is the main component and first component to be degraded to allow the invasion process. The ability to degrade and penetrate the basement membrane is related with an increased potential of the cells for invasion and metastasis. Cancers cells are able to produce MMPs that destroys the matrix barriers surrounding the tumour, including basement membrane, permitting invasion into connective tissues, entry and exit from blood and lymphatic vessels, and metastasis to distant organs. MMPs are family of zinc-dependent endopeptidases that collectively are capable of degrading all components of the ECM, including the basement membrane. Binding of breast cancer cells to type IV collagen in the basement membrane induces Discoidin domain receptor 1(DDR) gets activated and then it triggers signal transduction pathways and cellular processes that promotes secretion of MMPs which contributes to basement membrane degradation and cancer cell invasion.

In addition to MMP – 9 in the conditioned medium, the MDA – MB – 231 cells are also demonstrated to contain elevated level of signal transducer and activator of transcription 3 (STAT3) in the cells. STAT3 is typically maintained in the cytoplasm as an inactive monomer. Once it is phosphorylated, the STAT3 forms homodimers and enters into nucleus where it activates the transcription of multiple genes associated with cell proliferation and survival. The activation of STAT3 has been correlated with enhanced breast cancer cell growth, survival and immune evasion.[4]

Two chemokines, chemokine ligand 2 (CCL2) and chemokine ligand 5 (CCL5) are important. CCL2 is a potent chemo attractant for monocytes, memory T lymphocytes and natural killer cells whereas CCL5 is a potent inducer of leukocyte motility. Both chemokines stimulate migration of leukocytes in response to inflammatory signals. The roles of CCL2 and CCL5 in breast malignancy have been extensively addressed in breast cancer studies. Over expression of the CCL2 and CCL5 are stimulated during breast cancer development and progression. CCL2 and CCL5 act directly on the cancer cells to promote their pro-malignancy phenotype by increasing their migratory and invasion-related properties.[5]

Gardenia gummifera

Gardenia gummifera is a shrub about 1.8 M height, flowers are non odorous, a Calyx 1 Cm. Long Corolla at first, white and later changes to yellow. *Gardenia Lucida*, a large glabrous or small tree reaching 6 – 7.5 M height, young shoots, grayish green smooth resinous leaves 6.3 – 20 cm and flowers fragrant. The flowers open in the evening and changes color from white to yellow. It is geographically distributed in all districts of Tamil Nadu, Burma, Bangladesh, Konkan region, North Kanara and Malabar Coast.[6]

Ethanolic extracts of *Gardenia gummifera Linn*. possess twenty bioactive compounds viz. 1-Hexadecanol (17.53), Hexadecanoicacid, ethyl ester (11.25), Squalene (9.31), n-Hexadecanoic acid (7.57), Ethyl linoleate(6.91), Benzoicacid (5.43), Vitamin E (4.42), Phytol (2.94), 9,12-Octadecadienoicacid (2.93), Octadecadienoic acid, ethyl ester (2.76), Eicosanoic acid, ethylester (2.61), Corchoroside-B (2.49), Campesterol (1.83), 1-Heptatriacotanol (1.69),Neophytadiene (1.65), β-Sitosterol (1.05), Dodecane (1.02), Lucenin 2 (0.96), Quinicacid (0.93) andLoliolide (0.69).[7]

Dikamali is the gum resin obtained from the leaf buds of *Gardenia gummifera*. The resin is secreted in the form of tears. It is transparent greenish yellow in color with a sharp pungent smell.

Dikamali is known to have many medicinal properties like anthelmintic, antispasmodic, carminative, diaphoretic, expectorant, potentiating of pentobarbitone induced sleep, Anti-epileptic, peripheral and central Analgesic, Cardiotonic, Antioxidant, and Antihyperlipidemic. It is also useful in dyspepsia, flatulence for cleaning foul ulcers and wounds, and to keep off flies from wounds in veterinary practice. A number of flavanoids such as Gardenin A, B, C, D & E were isolated from Dikamali.[8]

Cycloartanes extracted from the *Gardenia gummiferais* found to possess anti-cancer and anti-HIV activities. This study was undertaken to screen the main cycloartane of Dikamali, Dikamaliartane-A for anti-cancer activity.

The gum resin of Dikamali cantains Acerosin, Apigenin, 3',4'-Dihydroxywogonin, 3',4'-Dimethoxywogonin, Gardenin A, B, E, 4'-Hydroxywogonin, 3-O-Methylkaempfero, Nevadensin, 5,7,3',4'-Tetrahydroxy-6,8-dimethoxyflavone, 5,7,3',5'-Tetrahydroxy-8,4'-dimethoxyflavone, 5,7,4'-Trihydroxy-6,8-dimethoxyflavone, 3',4',5'-Trihydroxywogonin, 3',4',5'-Trimethoxywogonin, Hexacosyl-p-coumarate, mixture of long chain (C_{22} – C_{26})esters, Dikamaliartane-A.[9]

Role of Cycloartane

Caspases play a central role in apoptosis initiation by inducing activation of caspase 3/7, 8. Their activities peaked at 18 hours for caspase 9 and 24 hours for caspase 3/7 and 8, which was then decreased. Pre-treatment with either caspase 8 or a pan-caspase inhibitor followed by the cycloartane resulted in higher cell viability than in cells without pre-treatment. However, inhibitors for caspase 3 and 9 did not rescue the cells from the cycloartane induced apoptosis. Cycloartane also shown that it has autoregulated effect on apoptosis signaling proteins like TNF -R1, FADD and TRADD proteins expression indicating that the compound induced the extrinsic apoptosis signaling pathway

through the TNF-R1 receptor. The mitochondrial-related pro-apoptotic proteins Bax and Bad also increased significantly. Cycloartane eventually reduces mitochondrial membrane potential, increased in cytochrome C release into the cytosol of cell line and NFkB translocation events.

Computational molecular docking illustrated the binding of the compound to TNF-R1 and revealed a total binding energy of -67.032 kcal. The binding site varies from the active site for the natural ligand, TNFα. The compound binds to the extracellular domain near the cell membrane. Hydrophobic interaction was reported between one of the cyclohexane and cyclopentane groups of the compound and cysteine – 96, and hydrogen bonds formed between the hydroxyl at C – 26 and carbonyl groups at C – 3 of the compound with lysine – 75 and 132 of the receptor, respectively.[10]

MATERIALS AND METHODS

Sample Collection

Leaves wear collected from the *Gardenia gummifera* from department of Horticulture, Ghandhi Krushi Vignana Kendra (GKVK) Bangalore.

Extraction procedure

Plant leaves were washed thoroughly with distilled Water. The leaves were dried under shade at room temperature. The dried leaves of *Gardenia gummifera* were finely grinded using electrical grinder and stored in air tight containers for further use. A total of 250 g of the pulverized plant material was extracted for 4 d in Methanol. The extracts were then filtered through Whatman's No. 1 filter paper and then condensed to dryness using rotary evaporator. The thick extracted mass was then dried at room temperature. Dried extract was collect.[11]

1. Phytochemical analysis

Phytochemical analysis of *Gardenia gummifera* leaves extracts were done using the protocols described by ; Segelman AB, Fransworth NR, Quimbi MD, L Loide for the following.[12]

Test for Sterols - Liebermann Burchard reaction
Tests for Alkaloids - Mayer's and Wagner's test
Tests for Tannins - Ferric chloride reagent test and
Tests for Saponins - Foam test
Tests for Phenols - Ferric chloride reagent test
Test for cardiac glycosides- Salkowiski test
Tests for Flavonoids
Test for Terpenoids

2. Hydroxyl radical scavenging assay

Principle

Hydroxyl radicals, generated by the Fenton type reaction system (Fe^{+3} + EDTA / H_2O_2 / Ascorbic acid), are known to damage deoxyribose and form TBA reactive chromogen, which forms a pink color measured spectrophotometrically at 532 nm.

Procedure

The deoxyribose assay is performed as described by Halliwell et al. with minor changes. The reaction volume of 1.0 ml contains 5.6mM deoxyribose, 2.8mM H_2O_2, 40 μM $FeCl_3$, 100 μM EDTA, and varying concentrations of the sample in 2.5mM phosphate buffer, pH 7.4. Initiation of the reaction is by the addition of 0.1mM Ascorbic acid. The mixture is then incubated for 90 minutes at 37°C. After incubation of 1ml of TBA (0.7% in 0.05 N KOH) and 1ml of 2.5% TCA the mixture is heated at 100°C for 8 minutes, cooled and the pink color formed is measured spectrophotometrically at 532 nm. Controls are to be run, which are devoid of test samples. Quercitin is used as the reference standard.[13]

3. HPLC analysis of Quercetin

Plant Extraction

10gms plant powder was extracted with 50ml Methanol at 50°C for 4 hours. The Methanolic extracts were filtered through Whatmann No. 1 filter paper and filtrate was evaporated to dryness. Methanolic extract (10mg/ml) was used for HPLC analysis.

Quercetin Standard: 100ug/ml prepared in Methanol.

HPLC Condition

Instrument: Shimadzhu LC- Prominence 20AT
Column : C18 column 250 mm x 4.6 mm, 5u particle
Mobile Phase: Linear
A : HPLC grade Acetonitrile (60%)
B : HPLC grade Water (40%)
Flow Rate : 1.0 ml/min
Injection volume: 10ul

Quantification of Quercetin in plant extracts

Concentration of Standard injected : 100µg/ml
Sample concentration : 10mg/ml

Formula used for quantification of quercetin in plant extract

Quercetin (Microgram/gram) = Sample area / Standard area X Standard concentration injected X Dilution factor.

4. Cytotoxicity studies using MDA-MB-231 cell line by MTT assay

MDA –MB – 231 cell line was obtained from American Type Culture Collection (ATCC) (Rockville, MD USA) (ATCC Number-HTB-26). The steps and procedure for cell culture, Thawing, Revival and Propagation of Cells were followed as described by Kangas, L. *et al.*[14]

Procedure

The collected cells should be about 70-80% confluency. Check the viability of the cells and centrifuge it. Take about 50,000 cells / well and seed it in 96 well plates and incubate for 24 hrs at 37^0C, 5% CO_2 incubator. Add plant samples which is to be tested from 0 – 320µg/ml (2 fold variation) concentration in RPMI without FBS & are incubated for 24 hr. Add 100µl/well of the MTT (5 mg/10ml of MTT in 1X PBS) to incubated plant samples

to the respective wells and incubated for 3to 4 hours. Discard the MTT reagent by pipetting without disturbing cells and add 100 µl of DMSO to rapidly solubilize the formazan. Measure the Absorbance at 590 nm.

Calculating Inhibition

% Inhibition = 100 – (OD of sample/OD of Control) X 100.

RESULTS AND DISCUSSION

1. Phytochemical analysis

Table 1: Phytochemical Analysis of*Gardenia gummifera*leaves extracts.

S.No	Tests	Observation	Inference
1	Froth formation test	Formation of stable froths was observed.	Presence of Saponins was confirmed.
2	Mayer's and Wagner's test	A brown color Precipitates was observed.	Presence of Alkaloid was confirmed.
3	Ferric Chloride test	Dark green color was developed.	Presence of Tannin was confirmed.
4	Liebermann-Burchard test	Formation of bluish green color was observed.	Presence of Steroid was confirmed.
5	Sodium hydroxide test	Change from yellow color to colorless was observed.	Presence of Flavonoid was confirmed.
6	Ferric chloride test	Violet color was developed.	Presence of Phenol was confirmed.
7	Salkowski test	Reddish brown coloration was observed.	Presence of Terpenoid was confirmed.
8	Benedict's test	Formation of an orange red precipitate was not observed.	Absence of reducing sugar was confirmed.
9	Biuret test	Formation of pink color in the extract layer was found.	Presence of protein was confirmed.

From the Table – 1, the qualitative analysis of *Gardenia gummifera* leaf extracts contains phytochemical compounds that include alkaloids, terpenoids, phenols, tannins, sponins, flavonoids, proteins and steroids except reducing sugars.

2. Hydroxyl radical scavenging assay

Table 2: Hydroxyl Radical scavenging assay.

Plants Name	Concentration (µg/ml)	Absorbance 546nm	% Inhibition	IC_{50}
Control	0.0	0.5934	0.00	
Standard (Quercitin)	6	0.5592	5.76	
	12	0.5143	13.33	
	25	0.4265	28.13	30.35
	50	0.2518	57.57	
	100	0.2158	63.63	
	200	0.1511	74.54	
Gardenia gummifera	3.12	0.5586	5.86	
	6.25	0.5118	13.75	
	25	0.4001	32.57	
	100	0.3018	49.14	30.98
	400	0.2589	56.37	
	600	0.1669	71.87	

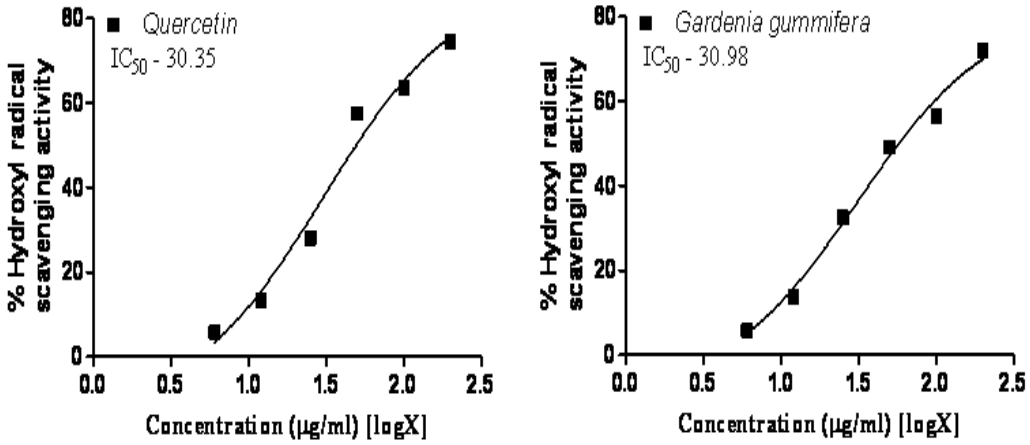

Figure 1: Hydroxyl Radical scavenging assay of Quercetin and *Gardenia gummifera.*

From Table – 2 and Figure – 1, the Hydroxyl radical scavenging assay of *Gardenia gummifera* leaf extract using Quercetin as standard IC_{50} (half maximal inhibitory concentration) values shows approximately same as Quercetin. Hence it is understood that the *Gardenia gummifera* (IC_{50}:30.98µg/ml) have same inhibitory concentration when compare to Quercetin (IC_{50}: 30.35ug/ml). It may also be due to presence of phytochemicals that exhibit Hydroxyl radical scavenging property.

3. HPLC analysis of Quercetin and*Gardenia gummifera*

Table 3: HPLC analysis of Standard Quercetin.

S. No.	Retention. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	1.933	373.177	22.881	20.5	14.0	0.20
2	3.107	92.433	3.352	5.1	2.0	0.49
3	3.487	1296.195	133.916	71.3	81.6	0.14
4	4.207	55.054	3.869	3.0	2.4	0.22
	Total	1816.859	164.018	100.0	100.0	

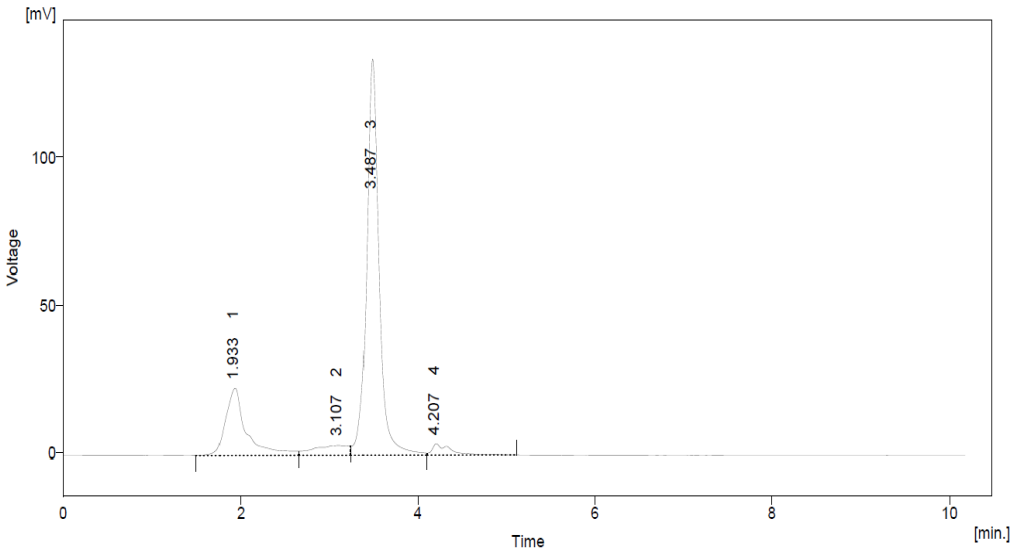

Figure – 2: HPLC analysis of standard Quercetin.

From Table – 3 and Figure – 4, the flavonoids wear quantified at 254nm using peak area by comparison with a calibration curve derived from the quercetin.

Table 4: HPLC analysis of Quercetin content in *Gardenia gummifera.*

S. No.	Retention Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	1.817	140.549	15.169	24.3	38.2	0.11
2	2.227	287.346	20.183	49.8	50.8	0.12
3	3.120	137.722	3.449	23.9	8.7	0.70
4	3.907	9.382	0.610	1.6	1.5	0.23
5	5.770	2.373	0.338	0.4	0.8	0.11
	Total	577.373	39.749	100.0	100.0	

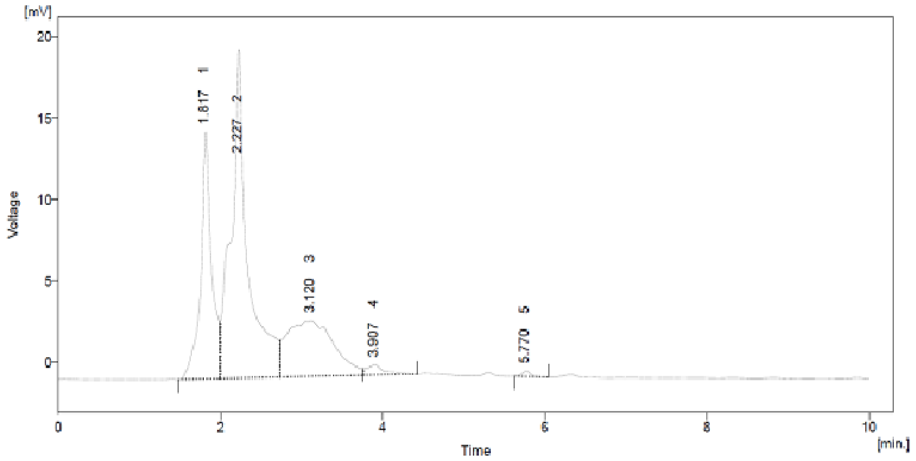

Figure 3: HPLC analysis of Quercetin content in *Gardenia gummifera.*

From Table – 4 and Figure – 3, the HPLC chromatograms from leaves of *Gardenia gummifera* the main difference was in peak eluted at 3.4min. External flavonoids were already analysed using HPLC method in various plant extracts. The peaks in this study shown marked decreased in peak area in case of *Gardenia gummifera* leaves when compared with standard quercetin.

From the calibration curve results, the amount of Quercetin, in the sample injected was calculated. *Gardenia gummifera* leaves contain no quercetin. Other peaks (#1) in both the HPLC chromatogram *Gardenia gummifera* leaves extracts indicated the presence of other chemical constituents The present method was applicable for determining quercetin in any aerial part of plant material using HPLC technique.

4. Cytotoxicity studies using MDA-MB-231 cell line by MTT assay.

Table 5: Cytotoxic study of *Gardenia gummifera.*

Plants name	Conc. µg/ml	OD at 590 nm	% Inhibition	IC_{50}
	Control	0.6538	0.00	
Gardenia gummifera	10	0.6225	4.79	69.03
	20	0.5752	12.02	
	40	0.5098	22.03	
	80	0.3861	40.95	
	160	0.2999	54.13	
	320	0.2272	65.25	

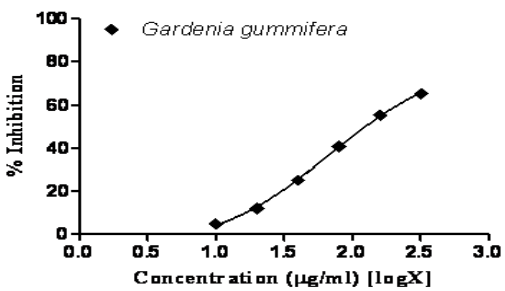

Figure – 4: Cytotoxic study of *Gardenia gummifera.*

From Table – 5 and Figure – 4, cytotoxic study of *Gardenia gummifera* on MDA – MB – 231 cancer cell line using MTT assay showed IC_{50} value was 69.03µg/ml. This suggests that plant extract showed significant inhibition of MDA – MB – 231 cell lines.

CONCLUSION

Our results in accordance with the above findings showed that *Gardenia gummifera* leaves possess maximum phytochemical components. The maximum

Hydroxyl Radical IC_{50} valve of standard Quercetin and *Gardenia gummifera* was found to be 30.35 and 30.98 respectively. The MTT assay states that it was found that there were cytotoxic effects with increasing concentration on MDA – MB – 231 cell lines from 10µg to 320µg concentration when compared to the untreated MDA-MB-231cells. These observations lend credibility that *Gardenia gummifera* leaves are good in radical scavenging activity.

ACKNOWLEDGEMENT

The authors are thankful to Skanda Life Sciences Private Limited, Bangalore for providing necessary facilities to complete this project successfully.

REFERENCES

1. Srivastava, J., Lambert, J., and Vietmeyer, N. 1996. "Medicinal Plants: An Expanding Role.
2. Parkin DM, Pisani P, Ferlay J. Global cancer statistics. CA Cancer J Clin, 1999; 49(1): 33-64.
3. Liu, H.; Zang, C.; Fenner, M.H.; Possinger, K. &Elstner, E. (2003) PPARgamma ligands and ATRA inhibit the invasion of human breast cancer cells in vitro. Breast Cancer Res. Treat, 79: 63-74.
4. Lu, X. & Kang Y. (2009) Chemokine (C-C motif) ligand 2 engages CCR2+ stromal cells of monocytic origin to promote breast cancer metastasis to lung and bone. J. Biol. Chem, 284: 29087-29096.
5. Selander, K.S.; Li, L.; Watson, L.; Merrell, M.; Dahmen, H.; Heinrich, P.C.; Muller-Newen, G. & Harris, K.W. (2004) Inhibition of gp130 signaling in breast cancer blocks constitutive activation of Stat3 and inhibits in vivo malignancy. Cancer Res, 64: 6924–6933.
6. Dolly Parmar Kotwa Innovative approach for assessing sustainability of the medicinal plant-Gardenia gummifera Linn. Journal of Horticulture and Forestry, 2014; 6(2): 14-21.
7. Kunert O, Sreekanth G, Sreedhar Babu G, Adavi Rao BV, Radhakishan M, Ravi Kumar B *et al*. Cycloartane Triterpenes from Dikamali, the Gum Resin of *Gardenia gummifera* and *Gardenia lucida*. Chem. Biodivers, 2009; 6: 1185-1192.
8. Krishnamurthi M, Seshadri TR, Sharma ND. Some Minor Components of Dikamali Gum. Indian J. Chem, 1971; 9: 189-190.
9. Sridhar Patwari, Harikiran L, Appa Rao AVN, Narsimha Reddy Y. Evalution of Anticancer Activity of Dikamaliartane-A, Cycloartane Isolated from Dikamali a Gum Resin. Int. Jorl of phar. and pharmaceutical, 4(4).
10. Kok Hoong Leong, Chung Yeng Looi, Xe-Min Loong, Foo Kit Cheat, Marc Litaudon. Cycloart-24-ene-26-ol-3-one, a New Cycloartane Isolated from Leaves of *Aglaia exima* Triggers Tumour Necrosis Factor-Receptor 1-Mediated Caspase-Dependent Apoptosis in Colon Cancer Cell Line. PLOS – One, 2016; 11(4).
11. Hemant RJ, Bhutani KK. Antioxidant properties of Indian Medicinal plants. Phytother. Res, 2002; 16(8): 771-3.
12. Segelman AB, Fransworth NR, Quimbi MD, L Loide. Biological and phytochemical evaluation of plants. Pub met, 1969; 1: 52-58.
13. Halliwell B, Gutteridge JMC and Aruoma OI. The deoxyribose method: a simple test tube assay-determination of rate constants for reaction of hydroxyl radicals. *Anal. Biochem*, 1987; 165: 215-219.
14. Kangas, L. *et al*. Bioluminescence of cellular ATP: A new method for Evaluating cytotoxic agents in vitro. *Med. Biol*, 1984; 62: 338–43.