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Qualitative and quantitative phytochemical analysis: *In vitro* studies of antioxidant and anticancer activity of *Bauhinia tomentosa* (L) leaf extracts

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Abstract
Herbal medicines are of great demand in both developed and developing countries, because of their enhanced bio efficacy and minimal side effects. In the present investigation, the leaf extracts of the plant *Bauhinia tomentosa* was evaluated for its cytotoxicity and antioxidant properties. The phytocompounds responsible for the bioactivity was analysed qualitatively and quantitatively. Phytochemical screening of the leaf extracts showed the presence of steroids, alkaloids, saponins, coumarins, flavonoids, phenols and amino acids. GC-MS analysis revealed the presence of nine major phytocompounds in the methanol leaf extract. Antioxidant efficacy of the leaf extracts was analysed *in vitro* by DPPH and superoxide radical assays and its cytotoxicity was evaluated on the MCF-7 cell lines. The leaf extracts were non-toxic and its potent antioxidant activity and anticancer activity may be due to the presence of phytocompounds such as flavonoids and phenols.

Keywords: Phytochemicals, antioxidant activity, cytotoxicity assay

1. Introduction
Medicinal plants have pharmacological activities and thus may be a source for novel anti-tumor agents. The mechanisms of action of anti-tumor agents include anti-proliferative and anti-oncogenic effects, induction of apoptosis, oncogenes and tumor suppressor genes. (Efferth, 2007) [1] Drug discovery from medicinal plants played an important role in the treatment of cancer and, indeed, most of the new clinical applications of plant secondary metabolites and their derivatives over the last half century have been applied towards combating cancer (Newman *et al*., 2000, 2003; Butler, 2004) [2, 3, 4].

Many plant extracts and phytoconstitutents are tried for their cytotoxic and anticancer potential and most of these plants tend to exert their anticancer properties through antioxidant mechanisms. (Renato moreira *et al*., 2007) [5] Antioxidants are compounds that, when added to food products, especially lipids and lipid containing systems, can increase the shelf life of the product by retarding the process of lipid peroxidation. Lipid peroxidation in fats and fatty foods not only brings about chemical spoilage in foods but also produces free radicals such as peroxyl and hydroxyl radicals, which are purportedly associated with carcinogenesis, mutagenesis and aging. (Nasr *et al*., 1996; Yagi, 1981) [6, 7]. Actually, most of the antioxidative potential of plant foods, which could be beneficial to human health, is due to phenolic compounds. Reactive oxygen species (ROS) are produced in all aerobic cells as by-products of oxygen metabolism. When ROS generation overwhelms the cellular antioxidant capacity, oxidative stress ensues. Under these condition ROS can oxidize lipids, proteins and nucleic acids, ultimately leading to cell death or transformation. Phenolic compounds acts as reducing agents, free radical scavengers, hydrogen donators and inhibitors of pro-oxidative enzymes (Cai *et al*., 2004; Gawlik-Dziki *et al*., 2012a) [8, 9] thus participating in the prevention of DNA adduct formation and enhanced carcinogen elimination. However, they can also exert chemopreventive effects through interference with ROS, which act as secondary messengers in signaling pathways crucial for cancer cell proliferation and invasion (Dai and Mumper, 2010) [10].

Bauhinia tomentosa Linn commonly known as yellow bell orchid tree belongs to *Caesalpiniaceae* family and is commonly known as kanchini in Tamil. In India and Sri Lanka, the root bark is used internally for conditions of the large intestine, while the flower is used as a remedy for dysentery and diarrhoea.

Leaves have antidiabetic action, all the part of the plant is recommended in combination with other drugs for the treatment of snake-bite and scorpion-sting (Sushruta) (Sammabamurthy, 2006) [11] (Raw material, 1988) [12] (Kirtikar and Basu 2006) [13].

2. Material and Methods

2.1. Preparation of plant extract

Bauhinia tomentosa leaves were collected and authenticated by Prof. P. Jayaraman, Plant Anatomy Research Centre (Voucher No: PARC/2015/3059), West Tamabaram, Chennai-45. The leaves were dried under shade at room temperature for about 20 days and powdered and sieved to get a fine powder using an electric blender. 70 g of the powder was filled in the thimble and extracted successively with petroleum ether, chloroform, ethanol and methanol using soxhlet extractor for 10 h. All the extracts were concentrated using rotary flash evaporator and preserved at 4^0 C in an airtight bottle for further use. (Fig.1) (Janaki *et al*., 2017) [14]

Fig 1: *Bauhinia tomentosa* Leaves

2.2. Phytochemical Screening

The qualitative phytochemical screening was carried out using standard procedures (Harbourne, 1998) [15] By this analysis, the presences of several phytochemicals were tested. Phyto chemicals were analysed quantitatively by HPLC

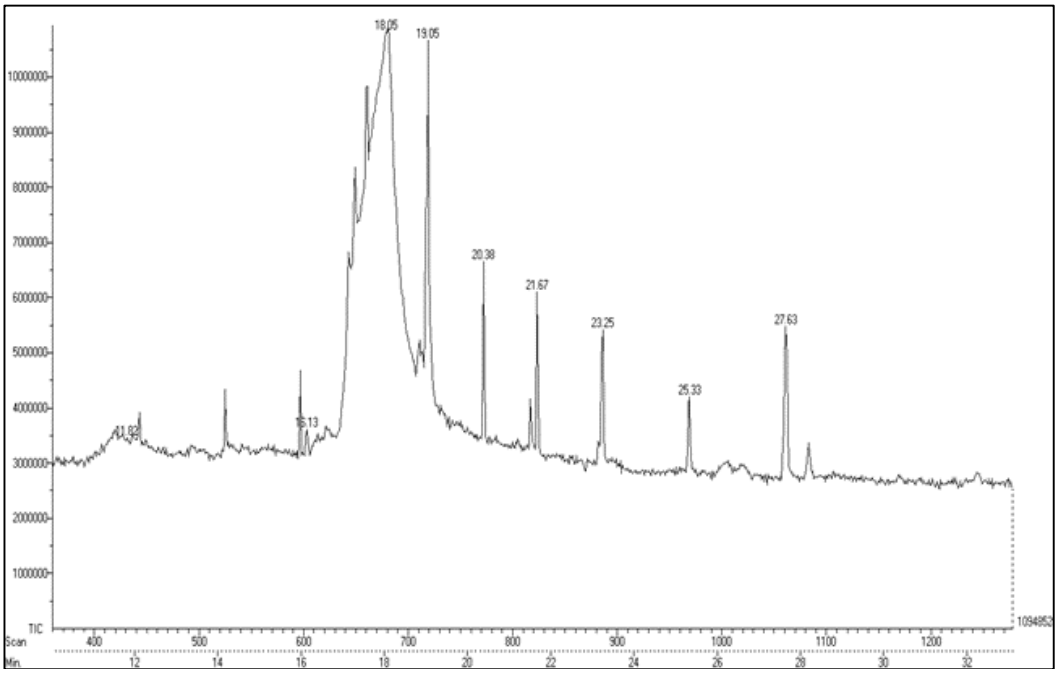

Fig 2: GC-MS analysis of methanol leaf extract

2.3. Antioxidant Assay

2.3.1. DPPH method

The 1,1-diphenyl-2-picrylhydrazine (DPPH) radical scavenging assay was first described by Blois (1958) [16] and was later modified slightly by numerous researchers. It is one of the most extensively used antioxidant assays for plant samples. DPPH is a stable free radical that reacts with compounds that can donate a hydrogen atom. This method is based on the scavenging of DPPH through the addition of radical species or an antioxidant that decolourizes the DPPH solution. The antioxidant activity is then measured by the decrease in absorption at 515 nm. In this method, 0.1 mM solution of DPPH in methanol is prepared, and 4 ml of this solution is added to 1 ml of the sample solution in methanol at varying concentrations. Thirty minutes later, the absorbance was measured at 517 nm. A large decrease in the absorbance of the reaction mixture indicates significant free radical scavenging activity of the compound (Blois, 1958) [16].

2.3.2. Superoxide radical antioxidant assay

The superoxide anion radicals are produced in 2 ml of phosphate buffer (100 mM, pH 7.4) with 78 µM - nicotinamide adenine dinucleotide (NADH), 50 µM nitro blue tetrazoliumchloride (NBT) and test samples at different concentrations. The reaction mixture is kept for incubation at room temperature for 5 min. It is then added with 5-methylphenazinium methosulphate (PMS) (10 µM) to initiate the reaction and incubated for 5 min at room temperature. The colour reaction between superoxide anion radical and NBT is read at 560 nm. Gallic acid is used as a positive control agent for comparative analysis. The reaction mixture without the test sample is used as control and without PMS is used as blank. (Nuno Rainha *et al*., 2011) [17] The scavenging activity is calculated as follows,

% scavenging activity = [(Control OD – Sample OD)/Control OD] × 100

2.4. GC–MS Analysis

GC–MS analysis were carried out an SHIMADZU QP 2010T which comprised of an autosampler and gas chromatography interfaced to a mass spectrometer (GC–MS) instrument employing the following condition: capillary column −624 ms (30 m × 0.32 mm × 1.8 m) operating in an electron mode at 70 eV; helium (99.999%) was used as carrier gas at a constant flow of 1.491 ml/min and injection volume of 1.0 ml, injector temperature was 140°C; ion source temperature of 200°C. The oven temperature was programmed from 45ºC. Mass spectra were taken at 70 eV.

2.5. Cell culture and cytotoxicity assay

The MCF-7 cell lines were procured from National Centre for Cell Science (NCCS), Pune, India. These cells were grown in Dulbecco's Modified Eagle's Medium DMEM supplemented with 2mM glutamine, 100U/ml penicillin, 100 µg/ml streptomycin and 10% fetal bovine serum (FBS). Cells were cultured in 75cm^2 cell culture flask at 37°C in a 5% CO_2 atmosphere. MCF-7 cells were cultured and seeded into 96 well plates approximately as 5x10^4 cells in each well and incubated for 24 and 48h. MCF-7 cells were treated with different concentrations of methanol extract (25µg/ml, 50 µg/ml, 100 µg/ml, 200 µg/ml, 400 µg/ml). After treatment, the plates were incubated for 24-48hrs in order to perform cytotoxic analysis. MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide,a yellow tetrazole) was prepared at a concentration of 5mg/ml and 10µl of MTT was added in each well and incubated for 4 hrs. Purple color formazone crystals formed were then dissolved in 100 µl of dimethyl sulphoxide (DMSO). The crystals were observed at 570nm in a multi well ELIZA plate reader. Optical density value was subjected to percentage of viability (Mossman, 1983) [18] (Prabhu *et al*., 2013) [19].

2.6. Statistical analysis

Three replicates of each sample concentration were used for statistical analysis by the Graph pad Prism version.5 software. Results with $P<0.05$ were considered to be significant.

3. Results

3.1. Phytochemical screening analysis of leaf extracts of *B. tomentosa*

In the present study, qualitative phytochemical screening of methanol, ethanol, chloroform, petroleum ether and aqueous extracts of the leaf of *Bauhinia tomentosa*. The methanolic leaf extract of *B. tomentosa* showed strong presence of saponin, alkaloids, flavonoids, steroids, phenols, coumarins and acids (Table 1). Quantitative phytochemical analysis of methanol leaf extract showed the presence of steroids 1.24 mg/g, alkaloids 0.44 mg/g, saponins 0.06 mg/g, coumarins 0.021 mg/g, flavonoids 1.44 mg/g, phenols 0.943 mg/g. (Table 2)

Table 1: Phytochemical screening of leaf extracts of *Bauhinia tomentosa*

S. No	Secondary metabolites	Aqueous	Ethanol	Methanol	Chloroform	Petroleum ether
1	Tannins	+++	++	-	+++	-
2	Saponin	+++	-	+++	-	++
3	Flavonoids	-	+++	+++	+++	-
4	Alkaloids	-	++	+++	+++	+
5	cyanins	+++	+++	+++	+++	+++
6	Quinones	-	-	-	-	-
7	Glycosides	-	-	-	-	-
8	Cardiac glycosides	+++	+	+++	++	++
9	Terpenoids	+++	+++	-	+++	-
10	Triterpenoids	-	++	-	++	-
11	Phenols	+++	+++	+++	+++	-
12	Coumarins	-	++	+++	+++	+++
13	Acids	++	+++	+++	+++	++
14	Proteins	-	-	-	-	-
15	Steroids	+++	+++	+++	+++	+++

+++Strongly positive++ Positive
+ Trace- Not detected

Table 2: Quantitative phytochemical analysis of *Bauhinia tomentosa* of methanol leaf extract

S. NO	Phytochemicals	Total Quantity mg/gm
1	Steroids	1.24
2	Alkaloids	0.44
3	Saponins	0.06
4	Coumarins	0.021
5	Acids	12.3
6	Flavonoids	1.44
7	Phenols	0.943

3.2. Antioxidant Assay - DPPH radical scavenging activity of leaf extracts of *B. tomentosa*

In vitro antioxidant activity of methanol, ethanol, chloroform, petroleum ether and aqueous extracts of *B. tomentosa* was measured on the basis of its DPPH scavenging activity. The 50 µg/ml concentration of methanol extract showed 74.42% scavenging activity followed by aqueous extract. (Fig.3)

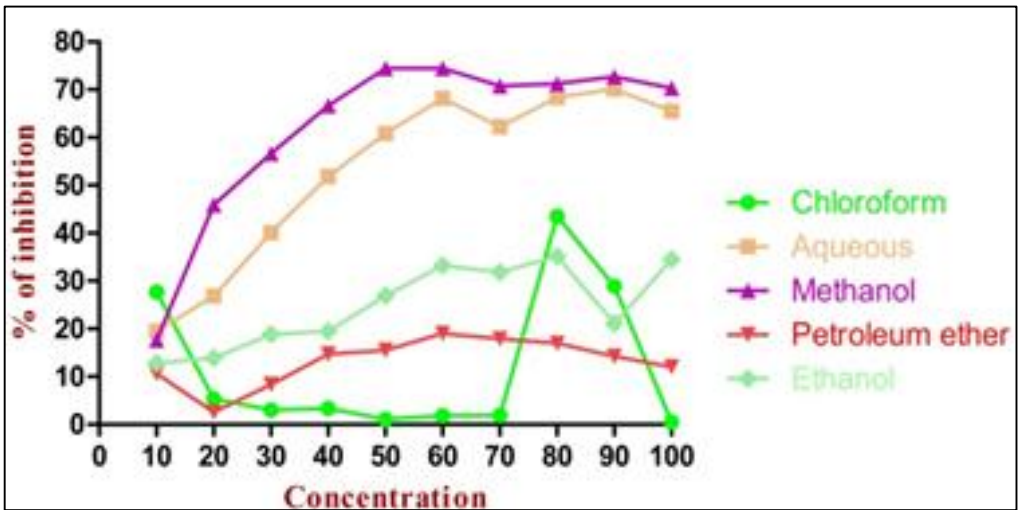

Fig 3: Antioxidant Assay-DPPH radical scavenging activity of leaf extract of *B. tomentosa*

3.3. Antioxidant Assay - Superoxide radical scavenging activity of leaf extracts of *B. tomentosa*

Antioxidant activity of methanol, ethanol, chloroform, petroleum ether and aqueous extracts of *B. tomentosa* was measured on the basis of its super oxide scavenging activity. The 100 µg/ml concentration of methanol extract showed 72.61% scavenging activity followed by ethanol extract. (Fig.4)

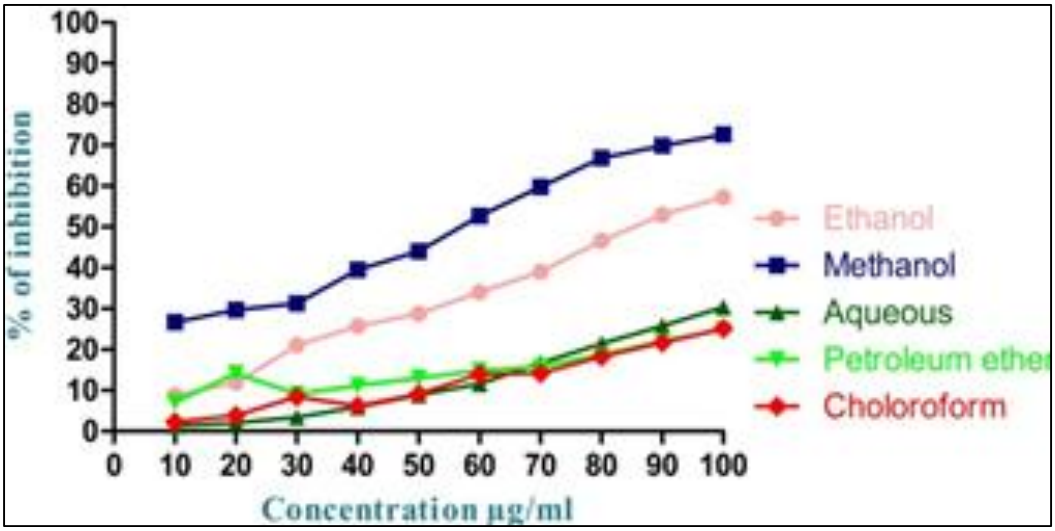

Fig 4: Antioxidant Assay-Superoxide radical scavenging activity of leaf extract of *B. tomentosa*

3.4. GC-MS analysis of methanol leaf extract of *B. tomentosa*

GC-MS analysis of the *B. tomentosa* revealed nine different compounds inmethanol leaf extracts. The major compounds are Z,E-2-Methyl-3,13-octadecadien-1-ol, Methyl abietate, Isopropyl stearate 2,6,10,14,18-Pentamethyl-2,6,10,14,18-eicosapentaene, Hexanedioic acid, 3-methoxy-,dimethyl ester, 1-Propen-3-one, 3-[o-acetylamino phenyl]-1-[p-methoxy phenyl], 2a,3b,5a,6a-T etramethoxycarbonyl- bicyclo [2,2,2] oct-7-ene, 2,4-Dimethoxy benzaldehyde phenylhy drazone, 1,3-Pentanedione, 4-methy l-1-pheny l. It was eluted at different rention time with large abundance. Among the nine components Isopropyl stearate showed a RT of 23.25 with 30.02% peak area. (Fig.2 Table.3)

Table 3: GC-MS analysis of methanol leaf extract

S. No	Retention Time	Compounds	Molecular Formula	Molecular Weight	Peak area%
1	19.05	Z,E-2-Methyl-3,13-octadecadien-1-ol	$C_{19}H_{36}O$	280.48	2.98
2	21.76	Methyl abietate	$C_{21}H_{32}0_2$	316.47	3.61
3	23.25	Isopropyl stearate	$C_{21}H_{42}0_2$	326.63	30.02
4	27.63	2,6,10,14,18-Pentamethyl-2,6,10,14,18-eicosapentaene	$C_{25}H_{42}$	342.60	24.77
5	16.13	Hexanedioic acid, 3-methoxy-,dimethyl ester	$C_9H_{16}O_5$	204.56	5.99
6	20.38	1-Propen-3-one, 3-[o-acetylaminophenyl]-1-[p-methoxyphenyl]	$C_{16}H_{15}NO_2$	253.29	8.83
7	25.33	2a,3b,5a,6a-T etramethoxycarbonyl-bicyclo[2,2,2]oct-7-ene	$C_{16}H_{20}O_8$	340.97	7.44
8	18.17	2,4-Dimethoxybenzaldehyde phenylhydrazone	$C_{15}H_{16}N_20_2$	256.30	5.42
9	11.82	1,3-Pentanedione, 4-methyl-1-phenyl	$C_{12}H_{14}0_2$	190.23	10.91

3.5. *In vitro* anti-cancer activity of methanol leaf extract of *B. tomentosa*

The methanol leaf extracts was evaluated for its effect on the cell viability against MCF7 cancer cell line at different concentrations. The methanol leaf extract showed a time and dose dependent decrease in cell viability. Increasing the time of incubation showed a further decrease in cell viability. Approximately 50% inhibition of cell viability was obtained with methanol crude extract at 182.38 µg/ml at 24 hours 93.73 µg/ml on 48 hours exposure. The results revealed that methanol extract were effective against MCF7 cells (Table.4) (Fig.5, 6).

Table 4: *In vitro* cytotoxicity of *Bauhinia tomentosa* methanol leaf extract against MCF7 cell line

S. No	Concentration	24 h	48 h
1	25µg/ml	25.63%	30.42%
2	50 µg/ml	35.67%	48.33%
3	100 µg/ml	48.07%	61.31%
4	200 µg/ml	60.73%	73.60%
5	400 µg/ml	75.42%	86.98%

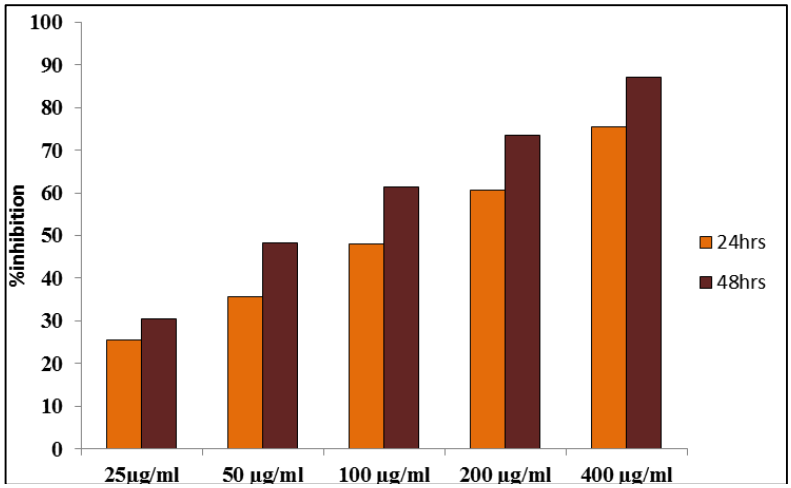

Fig 5: *In vitro* cytotoxicity of methanol extrat of *Bauhinia tomentosa* leaves

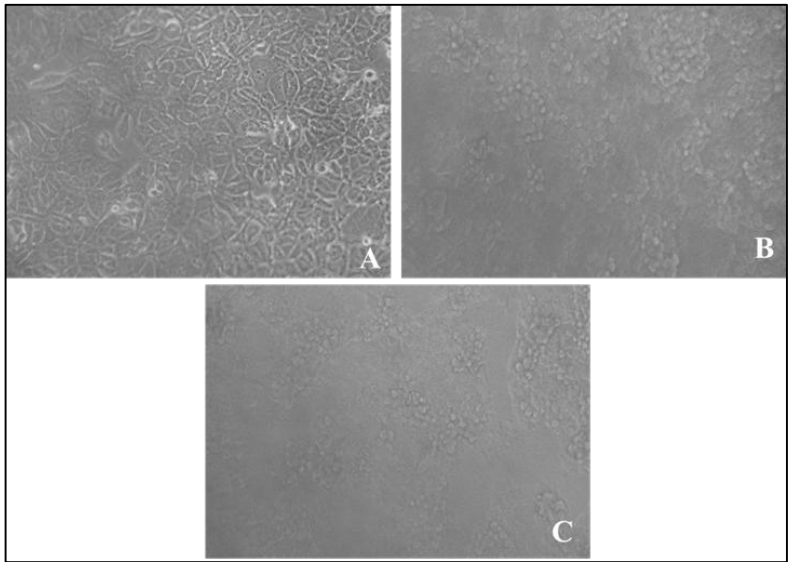

Fig 6: Morphology of control and treated MCF7 breast cancer cells (40x Magnification) Control; B) IC_{50} C) Maximum concentration (400 µg/ml)

4. Discussion

Plants produce two types of metabolites, primary metabolites are involved directly in growth and metabolism (carbohydrates, lipids and proteins), whereas secondary metabolites are considered as products of primary metabolism and are generally not involved in metabolic activity (alkaloids, phenolics, essential oils and terpenes, sterols, flavonoids, lignins, tannins, etc.) (Pal, 2007) [20] These secondary metabolites are the major source of pharmaceuticals, food additives, fragrances and pesticides, and herbicides (Okwu, 2005; Ramawat and Dass, 2009; Ramu and Mohan, 2012) [21, 22, 23]. Phytochemicals such as saponins have anti-inflammatory effects (Vinha and Soares, 2012) [24], haemolytic activity, and cholesterol binding properties (Nyarko and Addy, 1990) [25]. Glycosides are known to lower blood pressure (Marinkovic and Vitale, 2008) [26] and tannins exhibit antioxidant, antimicrobial and antiviral effects (Sayyah and Hadidi, 2004) [27].

The plant extracts revealed the presence of steroids, which are known to produce an inhibitory effect on inflammation, (Savithramma and Linga, 2011) [28] and alkaloids have been reported to exert analgesic, antispasmodic and antibacterial activities (Nyarko and Addy, 1990) [25] Phenols are among the non-enzymatic compounds obtained from natural sources, which have received high attention due to their proven antioxidant capabilities. Although phenolic compounds have been related to antioxidant activity, some studies have emphasized specific classes such as flavonoids and tannins to possess potent bioactivity (Gomes de Melo *et al*., 2010) [29]

The GCMS study of methanolic extract revealed the presence of esters, phenolics and steroids. These phytochemicals are responsible for various pharmacological functions like antimicrobial, anti-oxidant, and anti-inflammation activities. The gas chromatogram showed the relative concentration of various compounds eluted as a function of retention time. The heights of the peak indicate the relative concentrations of the components present in, *B. tomentosa* which is in accordance with compounds present in the extracts of *C. hirsutus* also (Meena *et al*., 2014) [30]

Antioxidants are known to inhibit oxidative stress. Reactive oxygen species (ROS), including free radicals such as superoxide anion radical (O-2), hydroxyl radical species (OH), singlet oxygen ($1O_2$) and hydrogen peroxide (H_2O_2) are active oxygen species that are often generated by biological oxidation reactions of exogenous factors These oxidative mediators can lead to the damage of biological structures such as nucleic acids, proteins and lipids. Many free radicals have been implicated in the causation of several diseases such as liver cirrhosis, atherosclerosis, cancer, diabetes, ageing and Alzheimer's disease (Kavitha *et al*., 2013) [31]

An integrated part of cancer cell development is the resistance to programmed cell death (apoptosis) and therefore re-establishment of apoptosis in cancer cells is a target mechanism for anticancer agents. (Josh *et al*., 1999) [32] Some plant-derived products are known to selectively induce apoptosis in cancer cells, which represent the ideal property for successful anticancer agents (Wamidh, 2011; Josh *et al*., 1999) [32, 33].

A cytotoxic compound induces loss of cell viability either by decreasing cell survival or triggering cell-death (Sumantran, 2011) [34]. The methanol leaf extract of *B. tomentosa* showed a time and dose dependent decreased cell viability in MCF7 cells. Increasing the time of incubation showed a further decrease in cell viability. Morphological changes of treated cells clearly indicates the play of apoptotic mechanisms leading to cell death which is evident by changes in membrane integrity inhibition of cell growth and cytoplasmic condensation and cell shrinkage appeared, similar results observed in fungal taxol on the cell viability in MCF7 cell lines for 24, 48 and 72 hours. (Vennila *et al*., 2012) [35] It showed that cell viability of control cells grew as irregular confluent aggregates with rounded and polygonal cell morphology after treatment appearance of polygonal cells that began to shrink and spherical in shape. (Vennila *et al*., 2012) [35]

Many of the agents that induce apoptosis are oxidants or stimulators of cellular oxidative metabolism, while many inhibitors of apoptosis show antioxidant activity (Buttke & Sandstrom 1994) [36]. Indeed, factors for oxidative stress, such as ROS production (Garcia-Ruiz *et al*.,1997; Coyle and Puttfarcken, 1993; Loo *et al*., 1993; Kruman *et al*., 1997; Albrecht *et al*., 1994) [37, 38, 39, 40, 41] lipid peroxidation (Hockenbery *et al*., 1993) [42], downregulation of the antioxidant defenses characterized by reduced glutathione levels (Marchetti *et al*.,1996) [43] and reduced transcription of superoxide dismutase, catalase, and thioredoxin, have been observed in some apoptotic processes (Briehl *et al*., 1996) [44] Moreover, ROS can also play an important role in apoptosis by regulating the activity of certain enzymes involved in the cell death pathway (Garcia-Ruiz *et al*.,1997; Coyle and Puttfarcken, 1993; Loo *et al*., 1993; Kruman *et al*., 1997; Albrecht *et al*., 1994) [37, 38, 39, 40, 41]. All these factors point to a significant role for intracellular oxidative metabolites in the regulation of apoptosis. Growth inhibition and ROS generation induced by *Antrodia camphorata* in MCF-7 cells indicates that ROS production was probably the cause of this apoptotic cell death, which is in accordance with the observations of the present study.

All the extracts which gave high antioxidant potency have higher anticancer activity. Our results are in line with earlier preliminary studies which showed a good relationship between antioxidant efficacy of plant extracts and anticancer potency.

5. Conclusion

Secondary metabolites of *Bauhinia tomentosa* leaves responsible for the higher antioxidant and anticancer activity. So, it could be new source for the development of therapeutic agents against cancer treatments.

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