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*Full Length Research Paper*

# Evaluation of retinoblastoma (Rb) and protein-53 (p53) gene expression levels in breast cancer cell lines (MCF-7) induced with some selected cytotoxic plants

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**Many Nigerian plants have been hypothesized to have anticancer potentials. However, not many of them have been subjected to acceptable scientific evaluation for their potential anticancer effects. In this study, six of such plants were selected to evaluate the effects of their crude, hexane, chloroform, ethylacetate, detanninfied and tannin fractions for brine shrimp lethality assay and the most cytotoxic fractions of each plant were further tested on gene expressions of TP53 and retinoblastoma (Rb) genes in human breast cancer cell line (MCF-7). *Gladiolus psittacinus* (Gps), *Icacina trichantha* (Itr), *Spilanthes filicaulis* (Sfi), *Curculigo pilosa* (Cpi), *Anthocleista djalonensis* (Adj), and *Tapinanthus bangwensis* (Tba) medicinal plants were selected for this study. Crude extracts of 80% aqueous ethanol macerated plant materials were fractionated into hexane, chloroform and ethylacetate fractions. The resultant aqueous fractions were detanninfied to produce aqueous detanninfied fractions and tannin fractions. The 36 panel of plant fractions produced from all the plants were used for the study. From our findings, hexane fraction of *S. filicaulis* (Sfi-HF) showed the highest cytotoxic effect ($LC_{50}$ 21.30 µg/ml) on brine shrimps showing a low signal of p53 gene expression but a high intensity of Rb gene expression in MCF-7 cell lines. Moreso, crude extract of *G. psittacinus* (Gps-CE) showed a significant ($P<0.05$) increase in TP53 gene expression in comparison with the control group and also a high intensity of Rb gene expression. Our results demonstrates the modulatory potentials of Sfi-HF and Gps-CE on TP53 and Rb gene expressions in MCF-7 breast cancer cell lines suggesting a possible mode of action of Sfi-HF and Gps-CE amongst a panel of 36 extract fractions.**

**Key words:** TP53 gene, retinoblastoma (Rb) gene, brine shrimps, cytotoxicity, gene expression, medicinal plants.

## INTRODUCTION

Among the various cancer types, breast cancer is the most common cause of cancer deaths. Statistics indicates that about 1.3 million women are diagnosed with breast cancer annually worldwide and about 465,000 die from the disease (Elangovan et al., 2008). The elucidation and molecular mechanisms underlying neoplastic transformation and progression have resulted in the understanding that breast cancer can be regarded as a genetic disease, which evolved from the accumulation of a series of acquired genetic lesions (Stoff-Khalili et al.,

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**Table 1.** List of medicinal plants used in this study.

| S/N | Scientific name | Common/Local name | Plants' part used |
|---|---|---|---|
| 1 | *Tapinanthes bangwensis* | Mistletoe/Afomo | Leaves |
| 2 | *Spilanthes filicaulis* | Brasil cress/Awere pepe | Leaves |
| 3 | *Gladiolus pscittanus* | Dragon head lily/Baaka | Bulbs |
| 4 | *Icacina trichantha* | False Yam/Gbegbe | Leaves |
| 5 | *Anthocleista djalonensis* | Cabbage tree/Ewesapo | Leaves |
| 6 | *Curculigo pilosa* | African crocus/Epakun | Rhizome |

2006). These genetic lesions lead to inactivation of tumor suppressor genes and/or activation of oncogenes (Stoff-Khalili et al., 2006).

An example of tumor suppressor activation in breast cancer is that of retinoblastoma (Rb1) (Lee et al., 1988), which is seen amplified and under-expressed in 10 to 15% of breast tumors, and in some series, has been associated with a worse prognosis or more aggressive clinical features (Osborne et al., 2004). TP53 is another tumour suppressor gene that functions to eliminate and inhibit proliferation of abnormal cells by inducing apoptosis and/or activating deoxyribonucleic acid (DNA) repair mechanisms, thereby preventing tumour/cancer development. TP53 gene has been shown to be the most commonly mutated gene in many common human cancers, with mutations estimated to occur in 50% of all cancers (Sigal and Rotter, 2000). Under normal cellular conditions, the p53 signaling pathway operates in ‘‘standby’’ mode. However, in the presence of cellular stresses such as DNA damage and expression of activated oncogenes, the signalling pathway is activated (Vogelstein et al., 2001). High levels of p53 protein are a common phenomenon in many human neoplasia (Vojtesek and Lane, 1993). Several studies have showed a close correlation between mutation of the TP53 gene and accumulation of high levels of p53 tumours (Troester et al., 2006; Vojtesek and Lane, 1993; Bennet et al., 1991; Davidoff et al., 1991). In early studies, expression of mutant p53 was demonstrated in breast cancer cell lines (Bartek et al., 1990).

However, studies have shown that TP53 gene is frequently inactivated by mutation or other mechanisms in human breast cancer, but only occurs in 30% of breast cancers (Alkahaf and El-Mowafy, 2003; Borresen-Dale, 2003; Olivier and Hainut, 2001). Gene expression *in vitro* studies have demonstrated that cell line models of luminal breast cancers show notable changes in $P_{53}$ genes such as p21, but the same magnitude of p53 regulated responses was not observed for cell line models of basal like breast cancer (Troester et al., 2006). Therefore, a luminal epithelial breast cancer cell line, MCF-7 was used in this study which is known to habour wild-type (normal) TP53 gene.

Bioactives from natural products are emerging strategies in suppressing tumours by inducing apoptosis (Alshatwi, 2011). Vinca alkaloids and taxoids are one of the earliest and major natural product derived drugs in mainstream cancer treatment (Warber et al., 2006). In addition, more phytochemical such as camptothecin and podophyllotoxin were discovered and many of these natural compounds were structurally modified to yield stronger anti-cancer analogues with less adverse effects (Azizi et al., 2009). In essence, it is important to investigate the mechanism of action of proposed antitumour phytochemicals in which understanding the modulation of gene expressions of oncogenes and tumour suppressor genes are key to tumour development. Based on published literatures and folklore in Nigeria, six plants (*Tapinanthes bangwensis* (Tba), *Spilanthes ficaulis* (Sfi), *Anthocleista djalonensis* (Adj), *Curculigo pilosa* (Cpi), *Icacina trichantha* (Itr) and *Gladiolus psittacinus* (Gps)) hypothesized to have antitumour properties were investigated for their cytotoxic activity on brine shrimps and modulatory property on p53 and Rb genes.

## MATERIALS AND METHODS

Plant materials used as listed in Table 1 were bought from Awolowo Market, a reputable herbal Market in Mushin Local Government Area of Lagos State, Nigeria. They were identified at the Herbarium Unit of Botany Department, University of Lagos, Lagos State, Nigeria, and voucher specimens were deposited.

### Preparation of plants' crude extracts

Plant materials were cut to increase surface area and were dried at 40°C for 3 days. After drying, 200 g of plants were pulverized and weighed in 500 ml 70% aqueous-ethanol to soak the pulverized plants. After 14 days of marceration, the residual plant materials (marc) were separated from the solvent by filtration over a muslin bag. The filtrates were further filtered using low ash filter papers. The crude extracts were concentrated using a hot-air oven at temperature of 45°C (Siedel, 2006).

### Organic solvents and column partitioning of crude extracts

The aqueous-ethanol extracts of the plant samples were individually fractionated by organic solvent extraction (n-hexane, chloroform and ethylacetate). Five grams each of concentrated crude extracts were dissolved in 20 ml deionized water and 70 ml of the organic solvent (n-hexane) was added and the immiscible

mixture was transferred into a separatory funnel. The resultant suspension was mixed vigorously and subsequently allowed to phase separate in order to separate the aqueous and organic fractions. After each separation, other organic solvents were sequentially added to the aqueous fractions for further partitioning into different organic solvents (Otsuka, 2006).

Polyphenolic compounds from the aqueous fractions were separated by passing 20 ml of 50 mg/ml aqueous fractions over 6 g polyamide column to trap the polyphenols. The column bound polyphenolic compounds were released by elution with 100 ml of absolute methanol (Collins, 1998).

#### Brine shrimps (*Artemia salina*) lethality assay

Brine shrimp cytotoxicity assay (Sowemimo et al., 2007) was used to screen the thirty six (36) fractions (crude extract, n-hexane, chloroform, ethylacetate, detannified, and tannin fractions for each of the six plants). One milliliter of each stock solution (50, 500, and 5000 µg/ml) was put into a test tube and made up to 5 ml with filtered sea water to give overall concentrations of 10, 100 and 1000 µg/ml. Ten brine shrimp larvae were then placed in each of the test tubes. After 24 h, the number of shrimps which survived were counted using a magnifying lens and recorded. All experimental assays were done in triplicates. The $LC_{50}$ was calculated using the Probit method (Wardlaw, 1985).

#### Cell culture and treatment

MCF-7 cell lines were grown in 7 Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal calf serum (FCS). Cells were incubated in a humidified 37°C, 95% $O_2$ + 5% $CO_2$ incubator and the medium was continuously changed every 2 to 3 days interval to feed the culture. At 70% confluence, $0.1 \times 10^5$ cells were seeded into 24 well plates in DMEM containing 2% FCS (maintenance medium). Seven fractions with the lowest brine shrimp lethality $LC_{50}$ values out of the thirty six (36) fractions (with at least one fraction representing each plant) were selected. A concentration that is five-fold lower than their $LC_{50}$ values were prepared in maintenance medium (inoculating medium). One milliliter of the inoculating medium was added to each well in replacement of the maintenance medium. The first three wells were used as the control (DMEM only) while the other wells contained inoculating medium of the selected fractions in triplicates.

#### RNA extraction and cDNA synthesis

At the end of 24 h incubation, cells were harvested and total RNA was extracted from each sample. The RNA was extracted according to Qiagen RNeasy kit (USA). Extracted RNA samples were quantified using Nanodrop spectrophotometer. 60 ng of normalized RNA samples were converted into cDNA in a reaction volume of 25 µl comprising of 500 ng oligodT, 1X Script buffer, 0.1 mM DTT, 1 U/µl Rnase inhibitor, 0.4 mM dNTP, 4.0 U/ul reverse transcriptase enzyme. PCR condition was done as described in the Jena Bioscience SCRIPT® Reverse transcriptase kit (Germany).

#### *Gene quantitation*

Polymerase chain reaction **(**PCR) was performed in a 25 µl reaction volume containing 5 µg of cDNA, 0.2 mM dNTP mix, 1X complete buffer (Jena Biosciences), 0.04 U/µl high yield taq polymerase (Jena Bioscience), 0.5X Sybr green I (Invitrogen, Germany) and 0.5 µM of each target primer pair. Thermal cycling was done at 94°C for 2 min; 94°C for 30 s; 56°C for 30 s; 72°C for 30 s; and 72°C for 2 min; step 2 to 4 was repeated 35 times. Primers pairs used in this study were *β-actin* fwd/rvs (5'-GGC ATG GGT CAG AAG GAT TC-3'/5'- ACA TGA TCT GGG TCA TCT TCT C-3'); *TP53* fwd/rvs (5'-GCG CAC AGA GGA AGA GAA TC-3'/5'-CAA GGC CTC ATT CAG CTC TC-3'); *Rb* fwd/rvs (AGGACCGAGAAGGACCAACT/AAGGCTGAGGTTGCTTGTGT). *β-actin* gene was used as the internal control (endogenous gene). PCR amplicons were run on 1.8% agarose gel electrophoresis in Tris-Acetate EDTA buffer at 120 V for 25 min. Gel images were captured and analyzed using Gelanalyzer.

#### Data analysis

The percentage lethality of the extracts and fractions on brine shrimps was calculated from the mean of larvae that survived in the treated tubes and controls. The numbers of dead nauplii was divided by initial number of nauplii (10) and multiply by 100. Finney's probit analysis was used to determine the $LC_{50}$ of each extract at 95% confidence interval.

Relative densitometric intensity of the PCR amplicon bands was done using Gelanalyzer software. The intensity values were computed into Graphpad Prism 5© software to calculate the significant differences between the control and test groups at 95% confidence interval.

## RESULTS AND DISCUSSION

The brine shrimp lethality test is a simple bioassay used for the primary screening of the crude extract of plant as well as isolated compounds to assess toxicity towards brine shrimp, which could also provide an indication of possible cytotoxic properties of the test materials (Meyer et al., 1982). A number of novel antitumor and pesticidal natural product have been isolated using this bioassay (McLaughlin et al., 1991). In this investigation, the extract and fractions of plants such as Tba, Sfi, Adj, Cpi, Itr and Gps were screened to assess their toxicity towards brine shrimps. After screening, the six most cytotoxic test agents with their respective $LC_{50}$ values out of the thirty-six are hexane fraction of Sfi (21.30), aqueous detanninfied fraction of Itr (66.08), aqueous detanninfied fraction of Adj (74.12), crude extract of Gps (82.68), hexane fraction of Tba (223.46), and crude extract of Cpi (274.20) as shown in Table 2. Bassey et al. (2012) reported that hexane fraction of Tba showed considerable cytotoxicity against brine shrimps.

Also, from the results, it was found that the crude fraction exhibited the highest cytotoxic activity for most of the plants, while the tannin fraction showed no significant cytotoxic activity for almost all the plants. The high cytotoxicity of the crude fraction might be as a result of a combination of bioactive compounds, both lipophilic and hydrophilic, present in the crude fraction, which probably enhanced its cytotoxic activity. On the other hand, the low cytotoxicity of the tannin fractions could be as a result of breakdown of tannins by the brine shrimps, which probably resulted in their low cytotoxicity. This is justified by the work of Makkar et al. (1995), who reported that invertebrates have the ability to secrete binding polymers

**Table 2.** Brineshrimp lethality assay of test fractions.

| S/N | Test fraction | $LC_{50}$ values on *Artemia salina* | | | | | |
|---|---|---|---|---|---|---|---|
| | | ***Icacina trichantha*** | ***Curculigo pilosa*** | ***Spilanthes ficaulis*** | ***Anthocleista djalonensis*** | ***Tapinanthes bangwensis*** | ***Gladiolus psittacinus*** |
| 1 | Crude extract (CE) | 185.68 | 274.20 | 30.87 | 148.78 | 993.78 | 82.68 |
| 2 | Hexane fraction (HF) | 1046.72 | 1640.65 | 21.30 | 154.74 | 223.46 | 4340.62 |
| 3 | Chloroform fraction (CF) | 319.47 | 964.57 | 444.30 | 426.15 | 904.78 | 475.46 |
| 4 | Ethylacetate fraction (EF) | 615.85 | 94037.10 | 51.57 | 103.65 | 864.98 | 114.52 |
| 5 | Aqueous detannified fraction (DF) | 66.08 | 479.75 | 646.54 | 74.12 | 1522.50 | 121.65 |
| 6 | Aqueous tannin fraction (TF) | 94037.10 | ND | 2046.28 | ND | 60519.18 | 140266.50 |

Lowest $LC_{50}$ values of each plant are shaded. ND= Not detected.

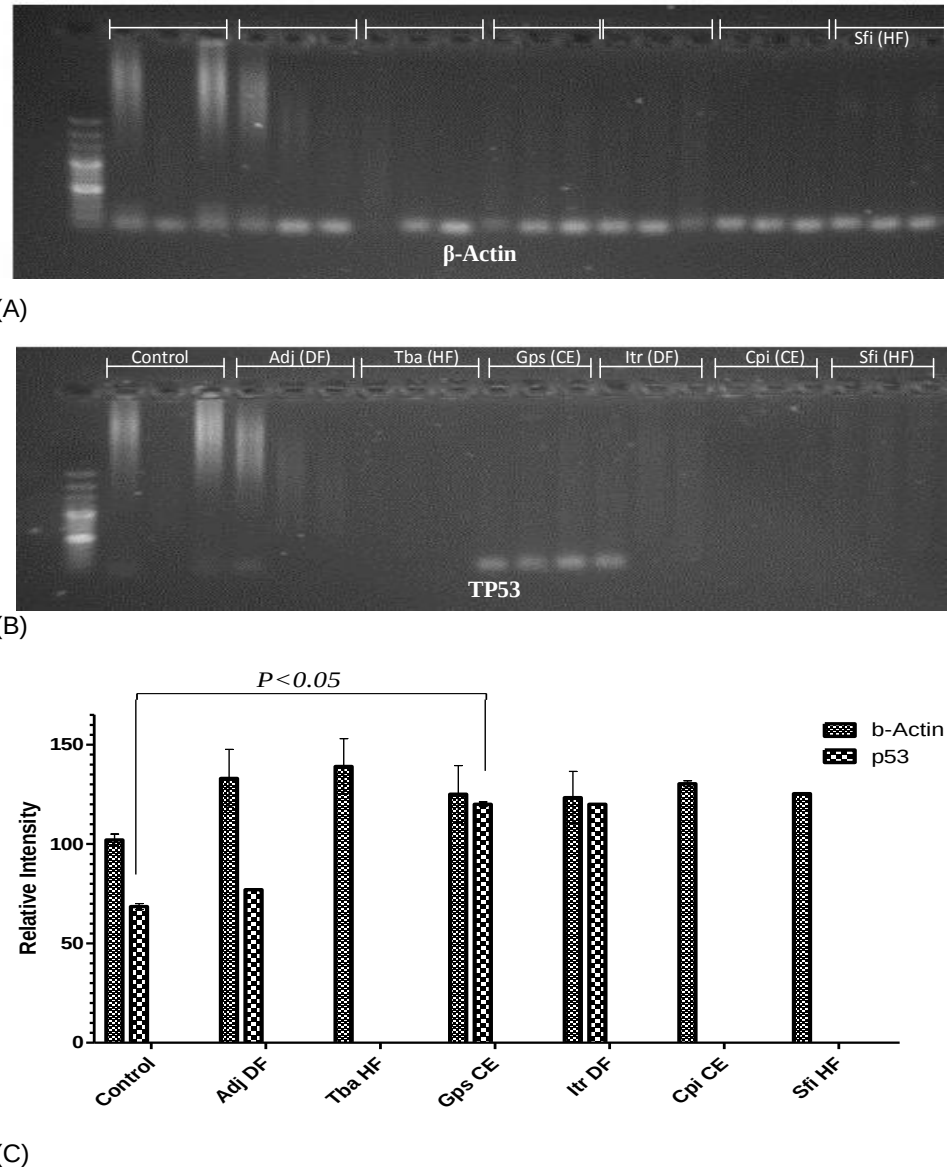
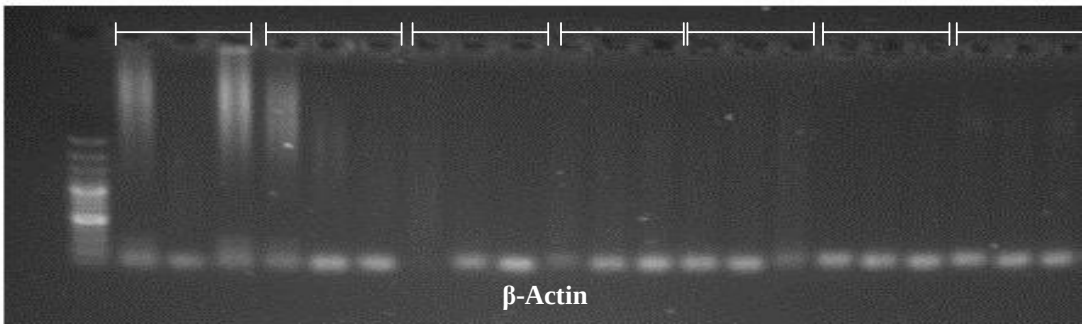
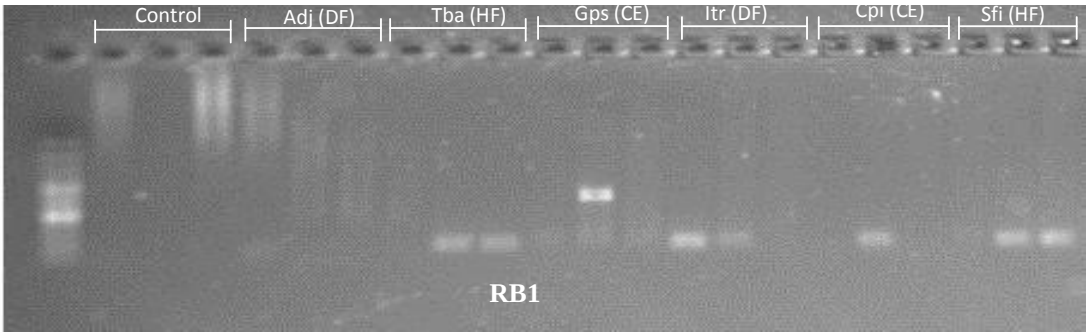


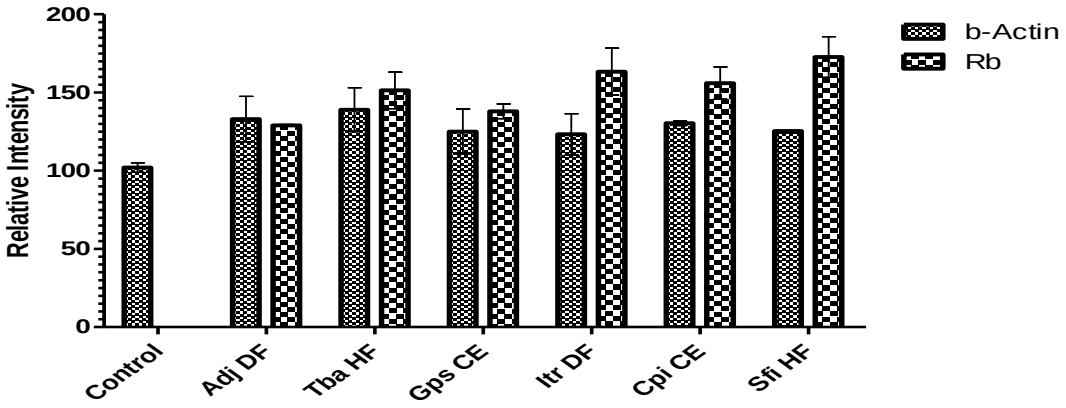

**Figure 1.** Densitometric analysis of RT-PCR amplicons for (A) β-Actin gene 1.8% agarose gel, (B) TP53 gene 1.8% agarose gel, and (C) error bar charts showing relative intensities of each band; testing was done in triplicates and values are Mean ± standard error (SE). P<0.05 is considered significant versus control group.




(A)




(B)




(C)

**Figure 2.** Densitometric analysis of RT-PCR amplicons for (A) β-Actin gene 1.8% agarose gel, (B) RB1 gene 1.8% agarose gel, (C) error bar charts showing relative intensities of each band; testing was done in triplicates and values are Mean ± standard error (SE). P<0.05 is considered significant versus control group.

and tannin-resistant enzymes as defense mechanisms against tannins which could lead to its biodegradation, thereby reducing its cytotoxic activity.

In the gene expression assay, the breast cancer cell line MCF-7 was treated with concentrations five-fold lower than the $LC_{50}$ values of each of the six most cytotoxic fractions selected for 48 h and the expression levels of TP53 and RB1 genes were evaluated. The retinoblastoma tumour suppressor gene (RB1) encodes a nuclear phosphoprotein that plays a central role in regulating the cell cycle in which its increase in expression levels can induce apoptosis (Weinberg et al., 1995; Reed, 2000). As shown in this study (Figure 2), all the cytotoxic fractions caused an upregulation of the RB1 gene with the hexane fraction of Sfi showing the highest followed by the aqueous detannified fraction of Itr. Retinoblastoma protein, pRb participates in a mechanism that arrests cell division in G1 when DNA damage is

detected. Unphosphorylated pRb binds the transcription factor E2F; while when bound to pRb, E2F cannot promote transcription of a group of genes necessary for DNA synthesis (DNA polymerase α, ribonucleotide reductase, thymidine kinase, proliferating cell nuclear antigen, and RAD51) (Nevins, 2001).

Several authors have investigated the effects of phytochemicals on TP53 gene expression as a measure of apoptosis induction in cell lines (Azizi et al., 2009; Yaacob et al., 2010; Wang and Sun, 2010; Alshatwi et al., 2011; Alshehri and Elsayed, 2012). It is well known that p53 acts biochemically as a transcription factor and biologically as a powerful tumor suppressor. Under normal, unstressed conditions, p53 protein remains undetectable due to its short half-life. The p53 instability is primarily controlled by its negative regulator Mdm2, which, as an E3 ubiquitin ligase, targets p53 for proteasome-mediated degradation (Wang and Sun, 2010). In this study, aqueous detanninfied Itr, Adj and crude extract of Gps caused upregulation of p53 gene as shown in Figure 1. Crude extract of Gps produced a significant ($P<0.05$) change in comparison with the control group. Samuel et al. (2011) reported the hepatoprotective ability of *Icacina trichantha* (Itr) sodium arsenite induced genotoxicity suggesting it as a possible anti-tumour agent. The observed increased expression in the treatment with *Itr*-DF, *Adj*-DF and *Gps*-CE suggests that the fractions contain bioactive compounds that restored the loss of function of p53 and Rb in the breast cancer cell line, MCF-7.

In conclusion, our results demonstrate the modulatory potentials of Sfi-HF on Rb and Gps-CE on both p53 and Rb gene expressions in MCF-7 breast cancer cell lines suggesting a possible mode of action of Sfi-HF and Gps-CE amongst a panel of 36 extract fractions.

## ABBREVIATIONS

**CE**, Crude extract; **HF**, hexane fraction; **CF**, chloroform fraction, **EF**, ethylacetate fraction; **DF**, aqueous detanninfied fraction; **TF**, tannin fraction.

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