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STUDY OF ANTICANCER ACTIVITY OF BRUSATOL, CURCUMIN AND PARAQUAT AGAINST CANCER CELL LINE THROUGH WESTERN BLOTTING STUDY ANALYSIS

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Abstract:

The present study scrutinized the anticancer activity of paraquat, curcumin, paraquat + curcumin; and brusatol + curcumin + paraquat against human colon adenocarcinoma cell line (Caco-2) and human pulmonary carcinoma cell line (NCI-H292). In adenocarcinoma cell line study, at the presence of curcumin, significant activation (+++) of Nuclear factor erythroid 2-related factor 2 (Nrf2) was noticed. In the combination of Brusatol + Curcumin + Paraquat, less activation (+) of Nuclear factor erythroid 2-related factor 2 (Nrf2) was observed. But in Pulmonary Carcinoma, at the combination of Brusatol + Curcumin + Paraquat, less activation (+) of Nuclear factor erythroid 2-related factor 2 (Nrf2) was observed. Commonly, in both cancer cell line, no activation (-) of Nuclear factor erythroid 2-related factor 2 (Nrf2) was seen in control. This study confirmed that the effect of anticancer of curcumin and its combinations.

Key Words: Curcumin, Paraquat, Brusatol, Western Blotting & Nrf-2 Activation

Introduction:

Curcumin from *Curcuma longa* suppresses and inverts carcinogenesis via multifaceted molecular targets. Several reports have demonstrated that curcumin inhibits animal and human cancers, suggesting that it may serve as a chemopreventive agent. Numerous in vitro and in vivo experimental models have also revealed that curcumin regulates several molecules in cell signal transduction pathway including NF-κB, Akt, MAPK, p53, Nrf2, Notch-1, JAK/STAT, β-catenin, and AMPK. Modulation of cell signaling pathways through the pleiotropic effects of curcumin likely activate cell death signals and induce apoptosis in cancer cells, thereby inhibiting the progression of disease (Shehzad and Lee, 2013).

Khor et al., (2011) demonstrated that curcumin reversed the methylation status of the first 5 CpGs in the promoter region of the Nrf2 gene in mice. The methylation DNA immunoprecipitation (MeDIP) analysis revealed that curcumin significantly reduced the anti-mecyt antibody binding to the first 5 CpGs of the Nrf2 promoter, corroborated the BGS results. Demethylation of Nrf2 was found to be associated with the re-expression of Nrf2 and one of its downstream target gene, NQO-1, one of the major anti-oxidative stress enzymes, both at the mRNA and protein levels. Taken together, their study suggests that curcumin can elicit its prostate cancer chemopreventive effect, potentially at least in part, through epigenetic modification of the Nrf2 gene with its subsequent induction of the Nrf2-mediated anti-oxidative stress cellular defense pathway.

Curcumin is demonstrated to induce remarkable antioxidant activity in a variety of cells and tissues. The study was aimed at identifying curcumin as a potent activator of nuclear factor erythroid 2-related factor 2 (Nrf2) and demonstrating its protective effect against inorganic arsenite- (iAs3+-) induced cytotoxicity in human keratinocytes. It was found that curcumin led to nuclear accumulation of Nrf2 protein and increased the expression of antioxidant response element- (ARE-) regulated genes in HaCaT keratinocytes in concentration- and time-dependent manners. High concentration of curcumin (20 μM) also increased protein expression of long isoforms of Nrf1. Treatment with low concentrations of curcumin (2.5 or 5 μM) effectively increased the viability and survival of HaCaT cells against iAs3+- induced cytotoxicity as assessed by the MTT assay and flow cytometry and also attenuated iAs3+- induced expression of cleaved caspase-3 and cleaved PARP protein. Selective knockdown of Nrf2 or KEAP1 by lentiviral shRNAs significantly diminished the cytoprotection conferred by curcumin, suggesting that the protection against iAs3+-induced cytotoxicity is dependent on the activation of Nrf2. The results provided a proof of the concept of using curcumin to activate the Nrf2 pathway to alleviate arsenic-induced dermal damage (Zhao et al., 2013).

Nrf2:INrf2 (Keap1) are cellular sensors of chemical- and radiation-induced oxidative and electrophilic stress. Nrf2 is a nuclear transcription factor that controls the expression and coordinated induction of a battery of defensive genes encoding detoxifying enzymes and antioxidant proteins. This is a mechanism of critical importance for cellular protection and cell survival. Nrf2 is retained in the cytoplasm by an inhibitor, INrf2 which functions as an adapter for Cul3/Rbx1-mediated degradation of Nrf2. In response to oxidative /electrophilic stress, Nrf2 is switched on and then off by distinct early and delayed mechanisms. Oxidative /electrophilic signals activate unknown tyrosine kinases in a pre-induction response that phosphorylates specific residues on Nrf2 negative regulators, INrf2, Fyn, and Bach1, leading to their nuclear export, ubiquitination, and degradation. This prepares nuclei for unhindered import of Nrf2. Oxidative/electrophilic modification of INrf2

cysteine 151 followed by PKC phosphorylation of Nrf2 serine 40 in the induction response results in the escape or release of Nrf2 from INrf2. Nrf2 is thus stabilized and translocates to the nucleus, resulting in a coordinated activation of gene expression. This is followed by a postinduction response that controls the "switching off" of Nrf2-activated gene expression. GSK3β, under the control of AKT and PI3K, phosphorylates Fyn, leading to Fyn nuclear localization. Fyn phosphorylates Nrf2 Y568, resulting in nuclear export and degradation of Nrf2. The activation and repression of Nrf2 provide protection against oxidative/electrophilic stress and associated diseases, including cancer (Niture et al., 2014). To carry the western blot analysis with paraquat, curcumin, paraquat + curcumin and brusatol + curcumin + paraquat using human colon adenocarcinoma cell line (Caco-2) and human pulmonary carcinoma cell line (NCI-H292).

Materials and Methods:

Curcumin:

Curcumin is a derivative from *Curcuma longa*. Curcumin powder was obtained from Sigma chemicals (St. Louis, MO, USA) and stored at room temperature. It was purchased as a crystalline solid. A stock solution was made by dissolving the curcumin in acetone. Further dilutions of stock solution into aqueous solution were made prior to performing experiments. Different doses of Curcumin were used (1, 5, 10, 25, 50 and 100 µg/ml).

Paraquat:

The herbicide paraquat (Paraquat Dichloride 24% SL) was purchased from HPM Chemicals & Fertilizers Ltd., Coimbatore and Tamil Nadu. Different doses of paraquat were used (1, 5, 10, 25, 50 and 100 µg/ml). Serial dilutions of the test herbicide were made as per the requirements in the present investigation.

Brusatol:

Brusatol(13,20-Epoxy-3,11β,12α-trihydroxy-15β-[(3-methyl-1-oxo-2-butenyl)oxy]-2,16-72dioxopicras -3-en-21-oic acid methyl ester) was purchased from Sigma chemicals (St. Louis, MO, USA). Required dilution was prepared using appropriate solvent. 1µg/ml dilution of brusatol was made as per the requirements in the present investigation.

Cancer Cell Lines:

The two test cancer lines (NCI-PBCF-HTB37 (Caco-2)- Human Colorectal Adenocarcinoma, (ATCC® HTB-37™) and (NCI-H292)-Human Pulmonary Carcinoma (ATCC® CRL-1848™) were procured from American Type Culture Collection (ATCC), Manassas, VA 20108 USA. Literature supplied along with these cell lines state that these cells were characterized by mycoplasma detection, DNA –Fingerprinting, isoenzyme analysis and cell vitality detection. These cells were maintained in cell culture media and conditions as per the recommendations of American Type Culture Collection centre.

Western Blotting:

The cells were treated as described in the figure legends. After the treatments, the cells were placed on ice, washed with cold PBS and lysed in RIPA lysis buffer that had been supplemented with 1 mM PMSF, a protease inhibitor cocktail and a phosphatase inhibitor cocktail. Cell lysates were centrifuged at 14,000 × g at 4°C for 5 minutes. Protein concentrations were determined using a BCA kit. Approximately 40–50 µg of cellular protein from each sample was loaded onto 8% or 12% SDS-polyacrylamide gels and electrotransferred to nitrocellulose (NC) membranes (0.22/0.45 µm). The blotted membranes were incubated with different primary antibodies, followed by incubations with secondary antibodies.

Co-Immunoprecipitation and Western Blotting:

For direct Western blot analysis, cells were lysed in lysis buffer (50 mMHepes, pH 7.6, 200 mMNaCl, 1 mM EDTA, 0.5% Nonidet P-40 supplemented with protease (10 µg/ml each of benzamidine, trypsin inhibitor, and bacitracin, 5 µg/ml each of leupeptin, pepstatin A, antipain, PMSF), and phosphatase (5 µM each of o-phosphoserine, o-phosphotyrosine, o-phosphothreonine, β-glycerophosphate, p-nitrophenylphosphate, and sodium vanadate) inhibitor cocktails). The lysate containing 50 µg of protein was separated by SDS-polyacrylamide gel. After electrophoresis, the gel was transferred to a nitrocellulose membrane. The protein of interest was visualized with chemiluminescence. The blots were re-probed with anti-α-actin antibody (Santa Cruz Biotechnology) to confirm equal protein loading.

Results and Discussions:

Western Blot Analysis - Colorectal Adenocarcinoma (NCI-PBCF-HTB37 (Caco-2) and Nrf-2 activation:

Treatment with paraquat showed less activation (+) of Nuclear factor erythroid 2-related factor 2 (Nrf2). In the presence of curcumin, significant activation (+++) of Nuclear factor erythroid 2-related factor 2 (Nrf2) was noticed. In the combined presence of paraquat and curcumin, again a significant activation (+++) of Nuclear factor erythroid 2-related factor 2 (Nrf2) was evident. In the combination of Brusatol + Curcumin + Paraquat, less activation (+) of Nuclear factor erythroid 2-related factor 2 (Nrf2) was observed. No activation (-) of Nuclear factor erythroid 2-related factor 2 (Nrf2) was seen in control.

Western Blot Analysis - Pulmonary Carcinoma (NCI-H292) and Nrf-2 Activation:

Treatment with paraquat showed less activation (+) of Nuclear factor erythroid 2-related factor 2 (Nrf2). In the presence of curcumin, significant activation (+++) of Nuclear factor erythroid 2-related factor 2 (Nrf2) was noticed. In the combined presence of paraquat and curcumin, again a significant activation (+++) of

Nuclear factor erythroid 2-related factor 2 (Nrf2) was evident. In the combination of Brusatol + Curcumin + Paraquat, less activation (+) of Nuclear factor erythroid 2-related factor 2 (Nrf2) was observed. No activation (-) of Nuclear factor erythroid 2-related factor 2 (Nrf2) was seen in control.

Curcumin is a powerful natural chemopreventive and anticancer agent. Its biological effects range from antioxidant, anti-inflammatory to inhibition of angiogenesis and is also shown to possess specific anti-tumoral activity. Curcumin has been shown to possess anti-angiogenic properties and the angioinhibitory effects of curcumin manifest due to down regulation of proangiogenic genes such as VEGF and angiopoitin and a decrease in migration and invasion of endothelial cells. One of the important factors implicated in chemoresistance and induced chemosensitivity is NFkB and curcumin has been shown to down regulate NFkB and inhibit IKB kinase thereby suppressing proliferation and inducing apoptosis. Cell lines that are resistant to certain apoptotic inducers and radiation become susceptible to apoptosis when treated in conjunction with curcumin. Besides this it can also act as a chemopreventive agent in cancers of colon, stomach and skin by suppressing colonic aberrant crypt foci formation and DNA adduct formation. This review focuses on the various aspects of curcumin as a potential drug for cancer treatment and its implications in a variety of biological and cellular processes vis-a-vis its mechanism of action (Singh et al., 2006).

Many chemicals that protect against carcinogenesis induce Nrf2-target genes. Such chemical agents induce the expression of genes that possess an antioxidant response element (ARE) in their regulatory regions. Under normal homeostatic conditions, Nrf2 activity is restricted through a Keap1-dependent ubiquitylation by Cul3–Rbx1, which targets the CNC-bZIP transcription factor for proteasomal degradation. However, as the substrate adaptor function of Keap1 is redox-sensitive, Nrf2 protein evades ubiquitylation by Cul3–Rbx1 when cells are treated with chemopreventive agents. As a consequence, Nrf2 accumulates in the nucleus where it heterodimerizes with small Maf proteins and transactivates genes regulated through an ARE. There is evidence for the existence of different classes of ARE (a 16-bp 5′-TMAnnRTGABnnnGCR-3′ versus an 11-bp 5′-RTGABnnnGCR-3′, with or without the embedded activator protein 1-binding site 5′-TGASTCA-3′), species differences in the ARE-gene battery, and the identity of critical Cys residues in Keap1 required for de-repression of Nrf2 by chemopreventive agents and phytochemicals from edible plants are known to induce Nrf2-target genes (Hayes et al., 2010).

Sakuma et al., (2014) found that curcumin inhibited Caco-2 cell proliferation through both an activation of the mitochondrial apoptotic pathway, and G2/M cell cycle arrest. Their findings provided increased insight into the mechanism of action of curcumin in colon cancer cells, and it may help researchers in this area to understand how this compound can protect against development of colon cancer. The cancer and proliferation pathways has demonstrated the preventive role of nuclear factor (erythroid-derived 2)-like 2 factor in case of oxidative stress which may otherwise lead to carcinogenesis. A detailed view of this protective factor is reviewed for a better insight of its defensive function in abnormal cell proliferation. Nuclear factor (erythroid-derived 2)-like 2 also known as NFE2L2 or Nrf2, is a transcription factor that in humans is encoded by the NFE2L2 gene. Nrf2 is a basic leucine zipper (bZIP) protein that regulates the expression of antioxidant proteins that protect against oxidative damage. Under normal or unstressed conditions, Nrf2 is kept in the cytoplasm by a cluster of proteins that degrade it quickly. Under oxidative stress, Nrf2 is not degraded, but instead travels to the nucleus where it binds to a DNA promoter and initiates transcription of anti-oxidative genes and their proteins. Nrf2 is kept in the cytoplasm by Kelch like-ECH-associated protein 1 (Keap1) and Cullin 3 which degrade Nrf2 by ubiquitination. Cullin 3 ubiquitinates its substrate, Nrf2.

Keap1 is a substrate adaptor, which helps Cullin 3 ubiquitinate Nrf2. When Nrf2 is ubiquitinated, it is transported to the proteasome, where it is degraded and its components recycled. Under normal conditions Nrf2 has a half-life of only 20 minutes. Oxidative stress or electrophilic stress disrupts critical cysteine residues in Keap1, disrupting the Keap1-Cul3 ubiquitination system. When Nrf2 is not ubiquitinated, it builds up in the cytoplasm, and translocates into the nucleus. In the nucleus, it combines (forms a heterodimer) with a small Maf protein and binds to the antioxidant response element (ARE) in the upstream promoter region of many antioxidative genes, and initiates their transcription. Activation of Nrf2 results in the induction of many cytoprotective proteins (Yamamoto et al., 2008 and Sekhar et al., 2010).

Kaspar et al., (2009) recorded that Nrf2:INrf2 (Keap1) are cellular sensors of chemical- and radiation-induced oxidative and electrophilic stress. Nrf2 is a nuclear transcription factor that controls the expression and coordinated induction of a battery of defensive genes encoding detoxifying enzymes and antioxidant proteins. This is a mechanism of critical importance for cellular protection and cell survival. Nrf2 is retained in the cytoplasm by an inhibitor, INrf2 which functions as an adapter for Cul3/Rbx1-mediated degradation of Nrf2. In response to oxidative/electrophilic stress, Nrf2 is switched on and then off by distinct early and delayed mechanisms. Oxidative/electrophilic modification of INrf2 cysteine 151 and/or protein kinase C phosphorylation of Nrf2 serine 40 results in the escape or release of Nrf2 from INrf2. Nrf2 is stabilized and translocates to the nucleus, forms heterodimers with unknown proteins, and binds the antioxidant response element, which leads to coordinated activation of gene expression. It takes less than 15 minutes from the time of exposure to switch on nuclear import of Nrf2. This is followed by activation of a delayed mechanism that

controls the switching off of Nrf2 activation of gene expression. GSK3β phosphorylates Fyn at an unknown threonine residue(s), leading to the nuclear localization of Fyn. Fyn phosphorylates Nrf2 tyrosine 568, resulting in the nuclear export of Nrf2, binding with INrf2, and degradation of Nrf2. The switching on and off of Nrf2 protects cells against free radical damage, prevents apoptosis, and promotes cell survival. The western blotting analysis proved that the combination of paraquat, curcumin, paraquat + curcumin; and brusatol + curcumin + paraquat are effective against human colon adenocarcinoma cell line (Caco-2) and human pulmonary carcinoma cell lines.

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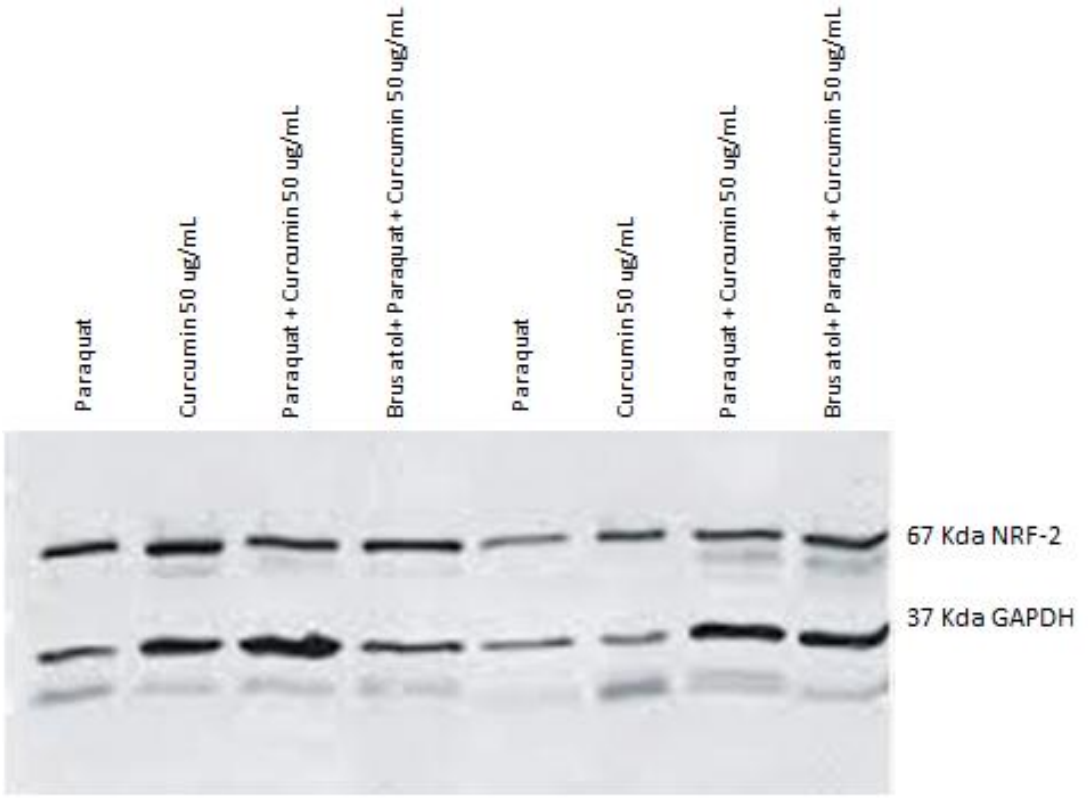

Figure 1: Representative photograph of the western blot experiment

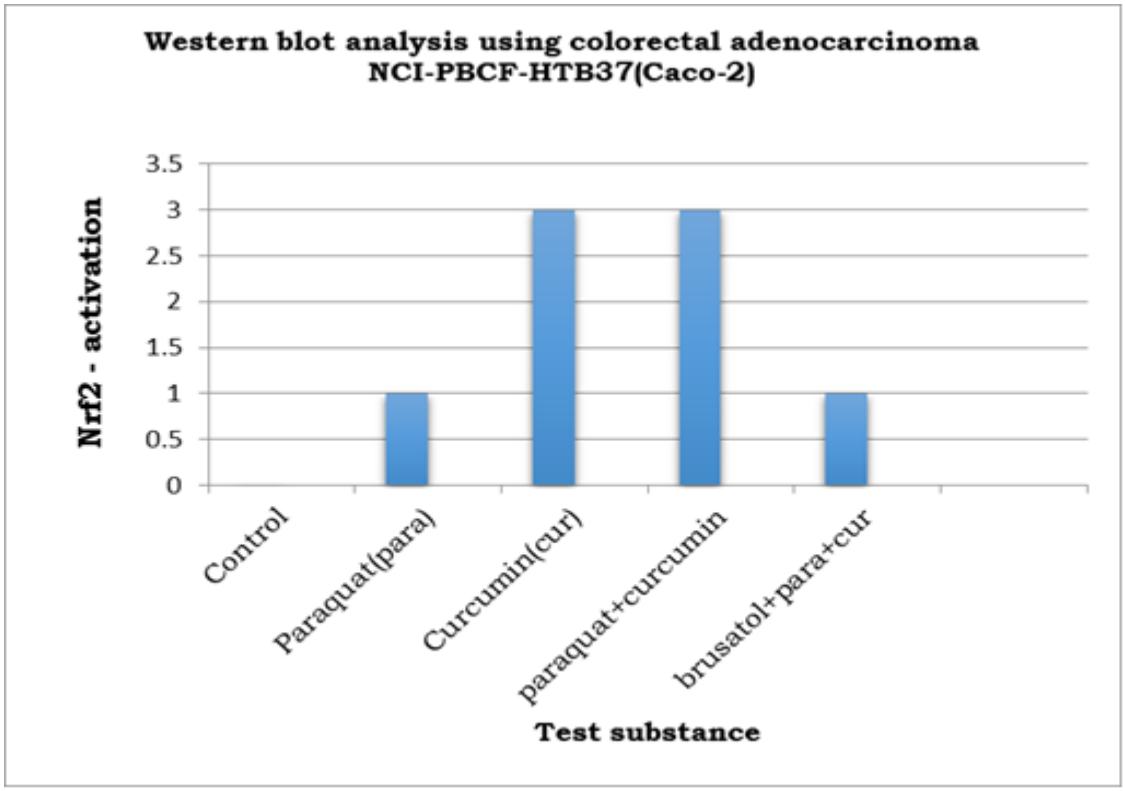

Figure 2: Western blot results using NCI-PBCF-HTB37 (Caco-2) cell line

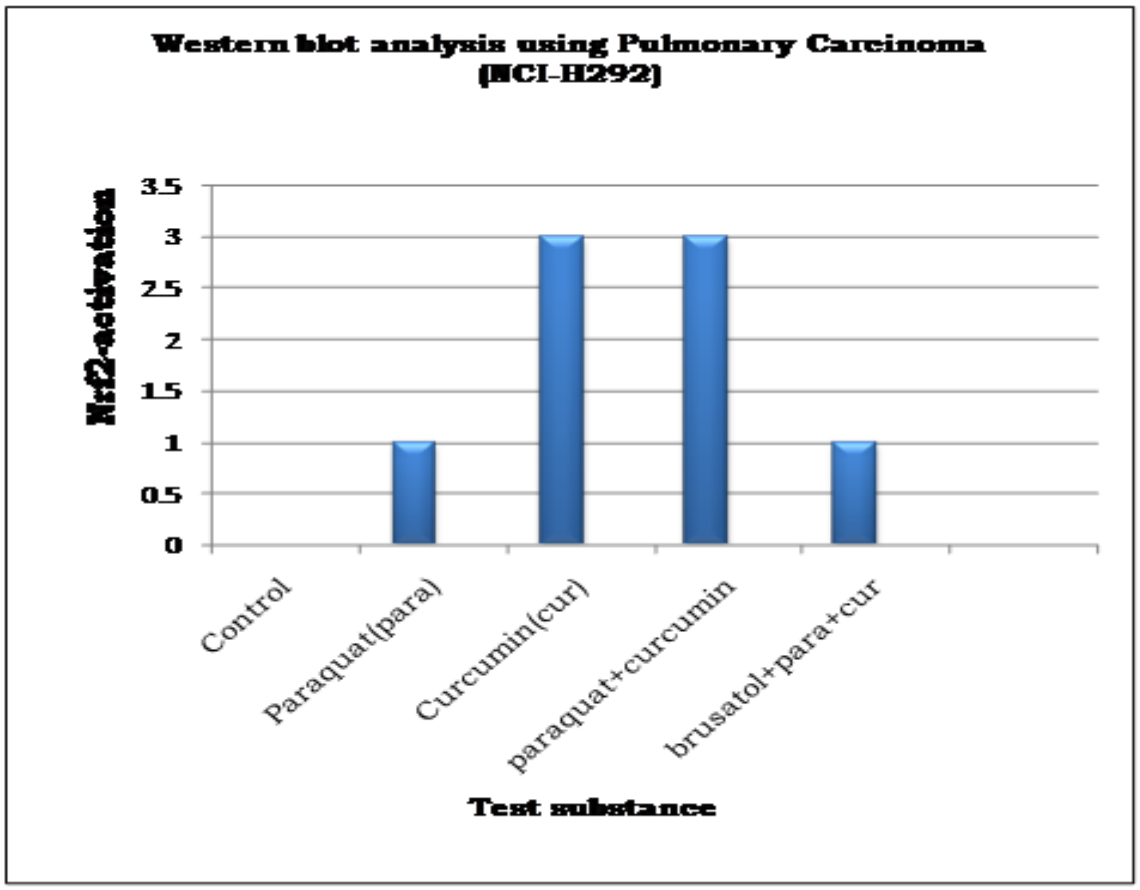

Figure 3: Western blot results using pulmonary carcinoma (NCI-H292) cell line