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In vitro cytotoxicity activity of the methanol leaf extract of *Picralima nitida* on Human Colorectal Adenocarcinoma Cell lines (HT-29)

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Abstract
The anti-proliferative effect of the methanol leaf extract of *Picralima nitida* on Human Colorectal Adenocarcinoma Cell lines (HT-29) was investigated using the Cell Titer 96 MTT assay because *Picralima nitida* is a therapeutic herb used in ethnomedicine for the management of several disease conditions. Cells were cultured to confluence, trypsinized, and plated in 96-well plates for cell proliferation assay. Twenty four hours after plating, cells were treated with various concentrations (62.5, 125, 250 µm) of the extract along with the control in the presence or absence of Calphostin C (10 µm) or Tyrphostin (10 µm) and cultured for 24–72 h to determine effects of treatment on cell growth. MTT assay was performed at 24, 48 and 72. MTT assay was performed over 3 days. *In vitro* antiproliferative study showed that the extract at all concentrations caused cytotoxicity of the HT29 cells. The extract in the absence of the enzyme inhibitors caused a higher cell inhibition than in their presence with the 62.5 µm/ml causing a higher inhibitory effect on the cells. The ability of the methanol leaf extract of Picralima nitida with a higher antiproliferative property in the absence of the enzyme inhibitors is a pointer to its cytotoxic efficacy. It was thus concluded from this study that the plant extract from *Picralima nitida* has anti-proliferative/cytotoxic effect hence further evaluation on the constituents responsible for this effect may be explored.

Keywords: Cytotoxicity, calphostin, tyrphostin, *Picralima nitida*

Introduction
It is a known fact that patients suffering from various cancer types are now supplementing their medical treatments with complementary treatment methods so as to reduce the symptoms associated with the side effects of these medical treatments and thus strengthen their immune systems [1, 2]. Complementary and Alternative Medicine (CAM) which involves different practices used in maintaining and improving health, preventing and treating diseases varies from country to country with respect to their use. The practices of CAM could either be alone or in combination with traditional medicine approaches [3]. The different treatment methods widely used in CAM include acupuncture, which has been shown to have ameliorative effects on the side effects such as nausea, vomiting, pain, poor sleep quality and anxiety experienced by cancer patients who were receiving chemotherapy [4, 5]. Hypnosis is another treatment method that may help reduce the symptoms of nausea and vomiting in patients with breast cancer, manage pain in a variety of contexts, and also reduce the anxiety level [6]. In fact, Saneei Totmaj *et al*. [7] has shown that ginger was able to cause reduction in nausea in breast cancer patients in acute phase of chemotherapy. In another study, turmeric and curcumin were able to reduce the intensity of erythema in the oral mucosa as well reduced pain in patients undergoing chemotherapy and radiotherapy [8]. The degree of lymphedema and pain in patients with lymphedema due to breast cancer were reduced significantly when cupping therapy was applied [9]. Phytotherapy has also been listed as one of the treatment methods used in cancer treatment [10]. In this study, the antiproliferative/cytotoxic property of *Picralima nitida* a therapeutic herb used for the management of many disease conditions in Nigeria was evaluated.

Picralima nitida Durand and Hook, (fam. Apocynaceae) is a West African plant that is distributed from Ivory Coast to West Cameroons, Congo basin and even Uganda [11]. It is used in ethnomedicine for the treatment and management of conditions such as malaria, abscesses, hepatitis, pneumonia, diabetes, and hypertension [12, 13].

The active phytochemical component of the plant extracts have been shown to have several pharmacological properties such as anti-inflammatory, analgesic, hypoglycemic, hypotensive, antiplasmodial, antimicrobial, antiulcer and antitumorigenic activities. Several studies have shown that phytochemicals such as glycosides, alkaloids, triterpenes flavonoids, polyphenols, saponins, and tannins are found in various extracts of this plant [12, 14, 19].

The antiproliferative or cytotoxic property of the methanol leaf extract of *Picralima nitida* (MLEPN) was thus evaluated in this study because it was said to possess antitumorigenic activity.

Materials and Methods

Plant collection and extract preparation

Fresh leaves of *Picralima nitida* were sourced from and identified at the School of Forestry, Jericho, Ibadan, Oyo state, Nigeria. The leaves of plant were cleaned with distilled water and air dried in a well ventilated shady room. The dried leaves were grinded to powder using blender and extracted in cold methanol in a screw-capped flask and shaken at room temperature. The solvent was filtered, squeezed off and evaporated off under reduced pressure in a rotatory evaporator at 40°C to obtain semi-solid crude extract which was stored at 4°C. The stored methanol extract of the plant was then used for the studies.

MTT assay

Cells were cultured to confluence, trypsinized, and plated in 96-well plates for cell proliferation assay. Twenty four hours after plating, cells were treated with various concentrations (62.5, 125, 250 µm) of the extract along with the control in the presence or absence of Calphostin C (µm) or Tyrphostin (10 µm) and cultured for 24–72 h to determine effects of treatment on cell growth. MTT assay was performed at 24, 48 and 72. MTT assay was performed over 3 days. On day one, after successful confluence, the HT29 cells were trypsinized, while on day two the cells were treated with the extract of *Picralima nitida* and the final volume of the media was adjusted to 100 µL and the incubation continued. On day three, 20 µL of 5 mg/mL of MTT was added to each of the 96 wells. The resulting mixture was then incubated for three and half hours at 37°C in a culture hood. After this, the media was carefully removed, 150 µL of MTT solvent was added and covered with tin foil and the cells agitated on orbital shakers for 15 min. Thereafter, the absorbance was read at 590 nm [20]. Percentage inhibition of the extract and the enzyme inhibitors were then determined using:

$$\% \text{ inhibition} = \frac{\text{Cell viability (Control)-Cell viability of test X 100}}{\text{Cell viability (Control)}}$$

Statistical Analysis

All values are expressed as mean ± S.D. The test of significance between two groups was estimated by Student's t test. One-way ANOVA with Tukey's post-test was also performed using GraphPad Prism version 4.00. The level of statistical significance was considered as $p < 0.05$.

Results

The results of the *in vitro* antiproliferative effect of the plant extract are as shown in Figures 1-4. In figure 1, the ability of the extract to cause cell agglutination in the presence or absence of Calphostin C and Tyrphostin was shown. The effect at 24, 48 and 72 h points were described in figures 2-4 respectively where the extract caused anti-proliferation effect on the cells with time indicating the cytotoxic property of this plant extract. For instance in Figure 2 in the control where the extract alone was treated with the cells, the 62.5 µg/ml concentration of the extract caused 52.6% inhibition while the 125 and 250 µg/ml caused 39 and 42.1% inhibition respectively. When the extract was incubated with Calphostin, the 62.5, 125 and 250 µg/ml concentrations caused 47.6, 45.2 and 42.9% inhibition respectively. On the other hand when the extract was incubated with Tyrphostin, the percentage inhibition for the 3 concentrations (62.5, 125 and 250) were respectively 51.3, 46.2 and 48.7%.

In figure 3 in the control experiment, the 62.5, 125 and 250 µg/ml concentrations caused 50, 42.9 and 28.6% inhibition respectively while the extract in the presence of Calphostin at these same concentrations respectively caused 8.3, 50 and 4.2% inhibition respectively. In the case of the extract in the presence of Tyrphostin, the percentage inhibitions for the 3 concentrations were 45.5, 45.5 and 36.4% respectively.

In Figure 4, the control experiment showed that while 62.5 µg/ml concentration of the extract caused 86.4% inhibition, the 125 and 250 µg/ml concentrations caused 81.8 and 84.1% inhibition respectively.

The result of the extract on the cells in the presence of Calphostin at 62.5, 125 and 250 µg/ml concentrations was 73.3% inhibition, while with Tyrphostin the percentage inhibitions for the 62.5, 125 and 250 µg/ml concentrations were 79.5, 82.1 and 76.9% respectively. The results showed that the least concentration i.e. 62.5 µg/ml concentration seemed to be more effective in causing cytotoxicity hence higher antiproliferative effect. Also when compared with the control, incubating the extract with the enzyme inhibitors i.e. Calphostin and Tyrphostin seemed to produce a lower inhibitory effect.

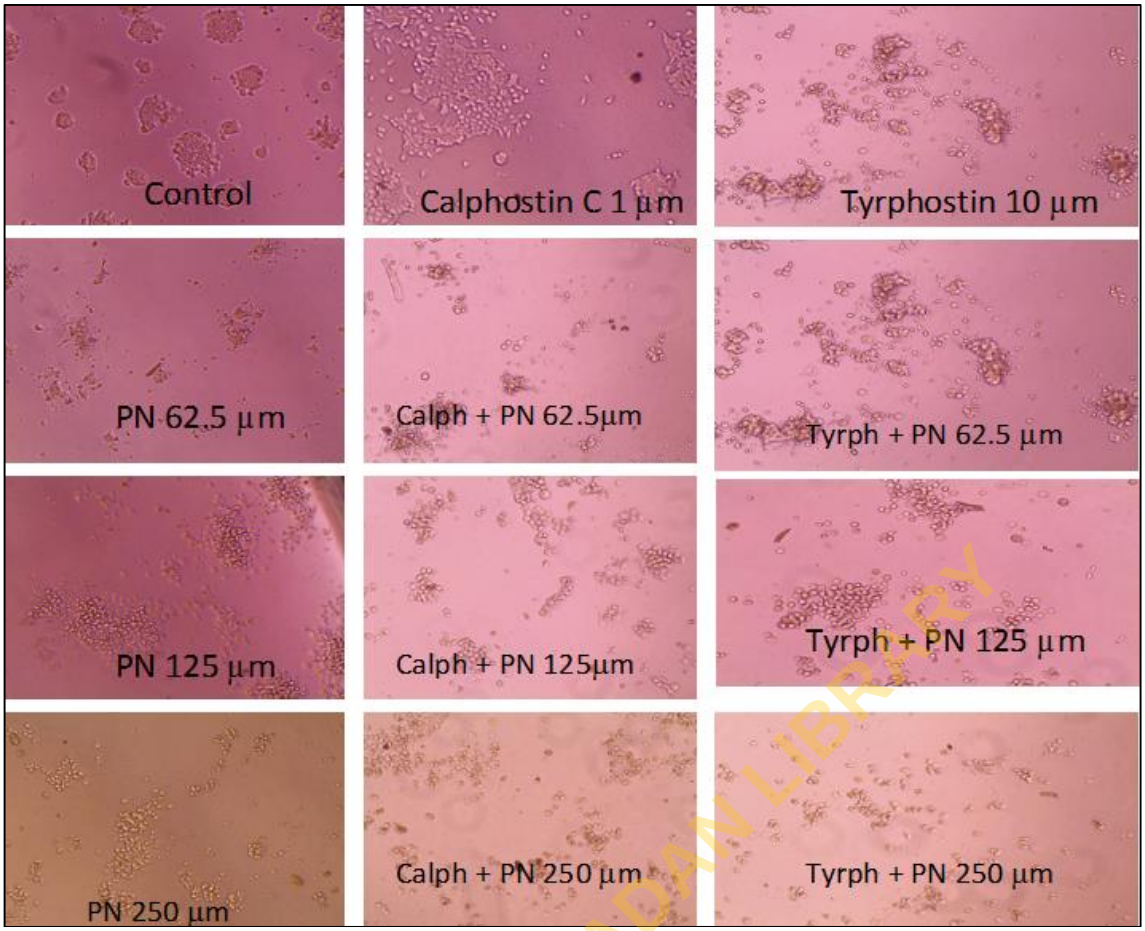

Fig 1: Effects of 24 h treatment with the methanol leaf extract of *Picralima nitida* in the presence or absence of Calphostin C or Tyrphostin on HT29

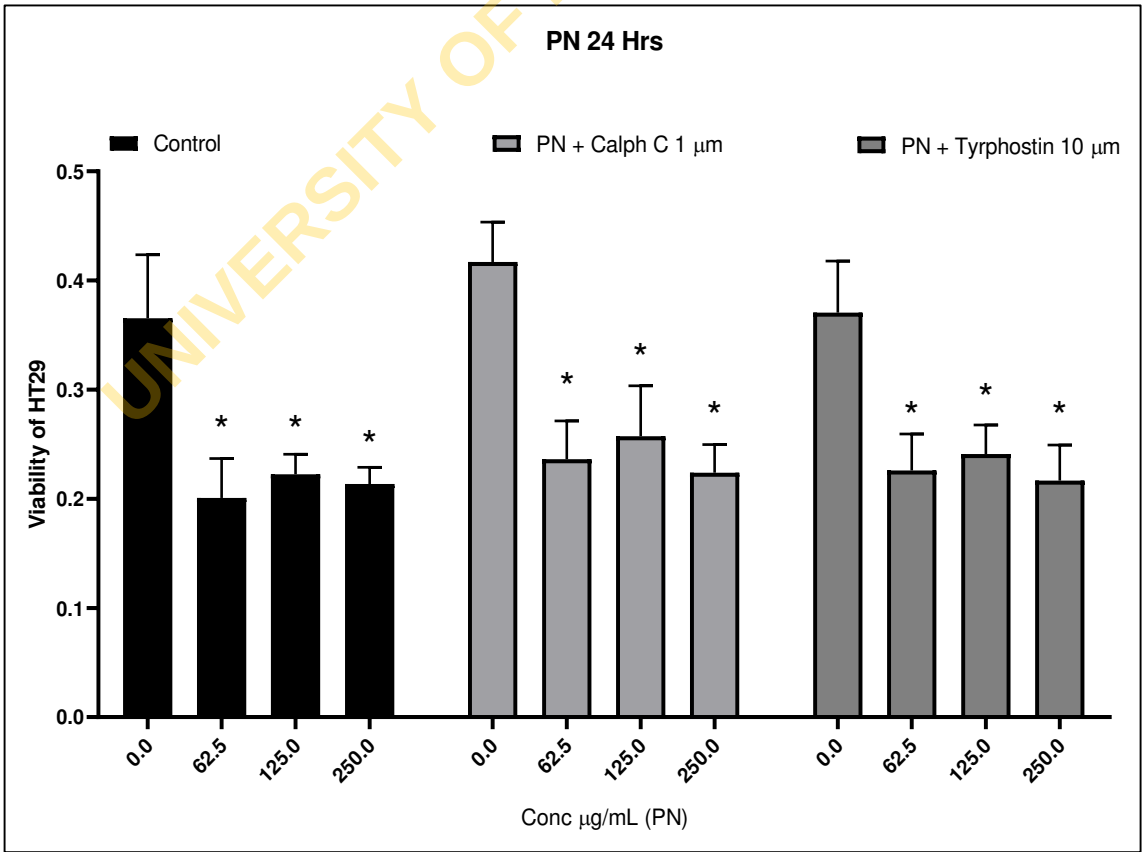

Fig 2: Effect of the methanol leaf extract of *Picralima nitida* (PN) on cell viability at 24-hour point in the absence or presence of Calphostin C and Tyrphostin

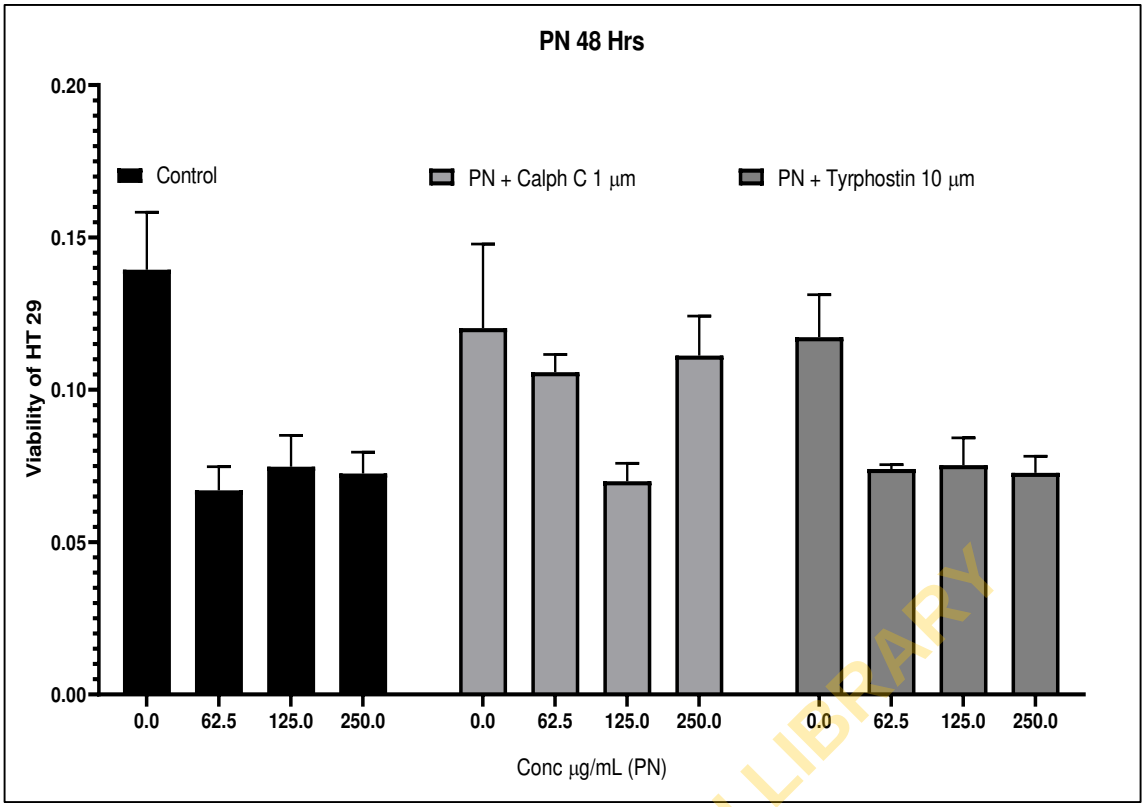

Fig 3: Effect of the methanol leaf extract of *Picralima nitida* (PN) on cell viability at 48-hour point in the absence or presence of Calphostin C and Tyrphostin

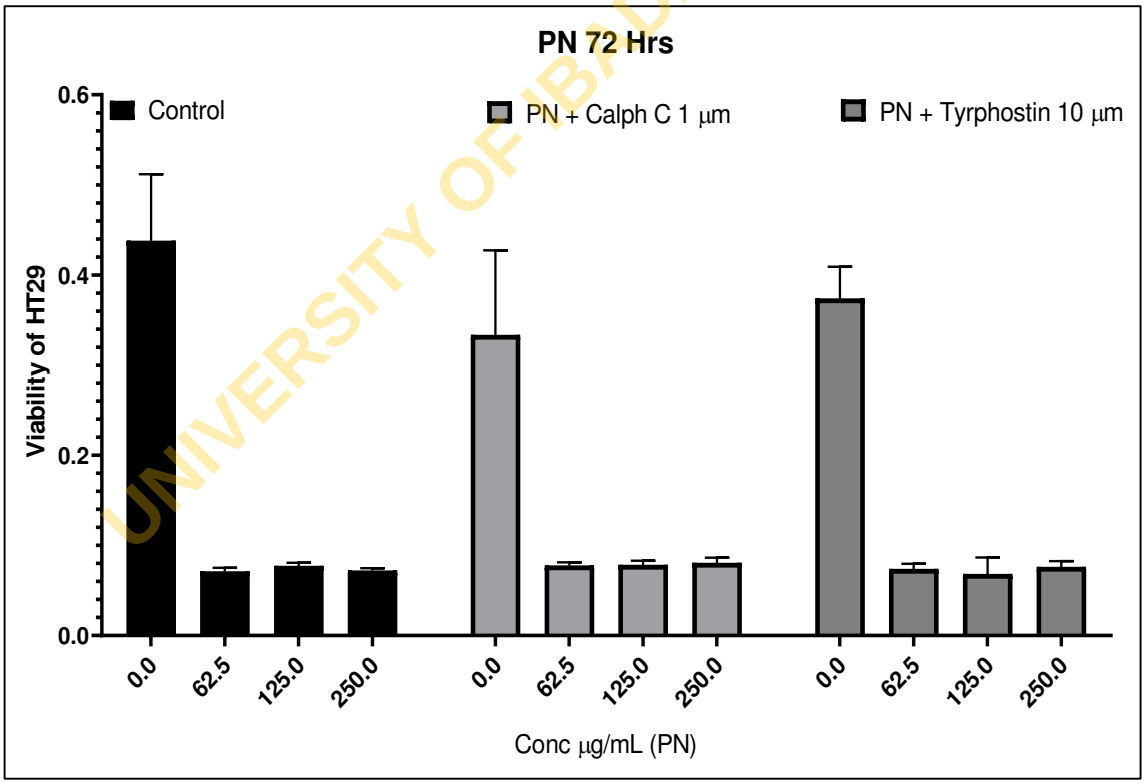

Fig 4: Effect of the methanol leaf extract of *Picralima nitida* (PN) on cell viability at 72- hour point in the absence or presence of Calphostin C and Tyrphostin

Discussion

The antiproliferative/cytotoxic property of the methanol leaf extract of *Picralima nitida* was investigated in the presence or absence of Calphostin C and Tyrphostin. The results showed that at 72-hour point, the antiproliferative effect of the extract was more pronounced especially when incubated with the cells alone. Although cell cytotoxicity was also experienced in the presence of Calphostin and Tyrphostin, the percentage cell inhibition was not as enhanced as when the extract was incubated alone with the cells. It was also discovered that the lowest concentration (62.5 μg/ml) of the extract showed the highest percentage inhibition on the average.

In vitro cytotoxicity testing forms part of the process required in the development of new biomaterials. These tests are

considered as screening assays, necessary for the evaluation of living cell's reactions especially in a cell culture assay, including cell viability and ability for cellular growth [21]. Although *in vitro* assays/methods cannot capture all complexities in the body during *in vivo* tests, all the same, they are an indispensable part of the evaluation process of new drugs candidate for example. These tests must always precede the *in vivo* tests because they are a valuable indicator of the potential behaviour of the newly developed biomaterial in contact with the tissue *in vivo* [22]. As a matter of fact, cytotoxicity is preferred as a pilot project test because it is an important indicator for toxicity evaluation of new drug candidate especially that it is simple, fast, has a high sensitivity and can save animals from toxicity [23]. It was in this respect that the methanol leaf extract of *Picralima nitida* was tested in an *in vitro* assay to determine their cell viability activity in Human Colorectal Adenocarcinoma Cell lines (HT-29) using MTT assay. An MTT assay is an *in vitro* colorimetric assay used in assessing the metabolic activity of the cell. The biochemical mechanism of this assay has to do with NAD(P)H-dependent cellular oxidoreductase enzyme that converts the yellow tetrazolium MTT [3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] into insoluble (E,Z)-5-(4,5-dimethylthiazol-2-yl)-1,3-diphenylformazan (formazan). This product, formazan could be dissolved with dimethyl sulfoxide (DMSO) leading to the formation of a purple color having its characteristic absorption value at 540 nm. It is the intensity of the purple color, which is directly proportional to the cell number, is that which determine the cell viability [24]. From this study, it is clear that the MTT assay used was suitable for determining the cell viability of the plant extract and the enzyme inhibitors. It has been reported that the aqueous and ethanolic leaf extracts of *P. nitida* leaf extracts are known to contain alkaloids, cardiac glycosides, saponins and terpenes along with strong larvicidal and antifungal properties [25]. These might have contributed to the pharmacological actions of this plant. For instance the antiproliferative and apoptotic effects of the crude methanol extract and fractions of the root bark of *P. nitida* root bark were investigated *in-vitro* using human breast cancer cell line (MCF-7) with the result showing a marked reduction in cell proliferation but increase apoptosis in MCF-7 cells after extract treatment. These antiproliferative and apoptotic effects were also shown to be highly significant ($P<0.001$) in the chloroform fraction of the extract [26]. In another study, the ethanol seed extract of *P. nitida* was shown to significantly ($P<0.05$) inhibit human T cell (Jurkat) proliferation activated by anti-CD3 antibody in a concentration-dependent manner (14). It may thus show that nearly every plant part and fraction of extracts of this plant has cytotoxic effect hence every effort should be made at discovering specific phytoconstituents responsible for this action.

Calphostin C is a potent inhibitor of the enzyme, protein kinase C [27] which has been known to act as a major receptor for the tumour-promoting phorbol esters [28] and this in turn activates a number of responses such as proliferation, survival, and motility hence influencing phenotypes associated with tumor progression and metastasis [29]. As a matter of fact during cancer cell proliferation and survival, PKC isozymes stimulate survival or proliferation-associated signaling pathways, such as Ras/Raf/MEK/ERK or PI3K/Akt (also known as PKB)/mTOR pathways, however it suppresses the expression of cancer suppressor-associated or apoptotic signals such as caspase cascade or Bax subfamily [30].

Tyrphostin AG957 on the other hand is a tyrosine kinase inhibitor with anti-BCR/ABL tyrosine kinase activity [31] where it causes dose-dependent growth inhibition with one of its therapeutic value is in cancer/cell hyperproliferation [32] due in part to its ability to reduce free radical production in mitochondria [33], as well as activation of Nrf2 transcription factor [34, 35].

In the absence of these enzyme inhibitors, the extract inhibited cell proliferation and in their presence the anti-proliferation of HT29 was established though there appeared to be a slight reduction in the antiproliferative effect of the extract in the presence of these enzyme inhibitors. It may however showed that the extract may have acted in the same pathway as these enzyme inhibitors that also have their therapeutic indication as anticancer agent.

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