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Securinega virosa (Euphorbiaceae) root bark extract inhibits glioblastoma multiforme cell survival *in vitro*

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Securinega virosa **Roxb (Ex Willd) is a commonly used medicinal plant in the management of inflammatory conditions and tumor in African traditional medicine. In this study, the effects of the crude methanol root bark extract and its fractions were evaluated on the survival of Glioblastoma mutiforme (GBM) tumor cells (U-1242) *in vitro*. The effects of the crude extract and its fractions on epidermal growth factor receptor (EGFR) which is overexpressed in about 40 to 60% of GBM patients were also evaluated. Their activities on the phosphorylation of downstream mediators of tumor cell growth and proliferation such as Akt and mitogen activated protein kinase (MAPK) were also studied using western blot analysis. The crude methanol root bark extract and the various fractions; namely, residual aqueous, n-butanol soluble and chloroform fractions significantly ($P < 0.0001$) and concentration-dependently (31.25 to 500 µg/ml) reduced tumor cell growth with IC_{50} ranging from 4.87 µg/ml for the chloroform fraction to 58.5 µg/ml for the butanol fraction. The crude methanol root bark extract and the residual aqueous fraction blocked EGFR phosphorylation at tyrosine 1068 and 1045 sites. They also significantly inhibited platelet derived growth factor receptor (PDGFR) phosphorylation. In addition, crude methanol root as well as the residual aqueous and butanol fractions blocked phorbol myristate acetate (PMA) and PDGFR-induced transactivation of the EGFR at tyrosine 1068. Similarly, the crude methanol root bark extract reduced the phosphorylation of MAPK. In contrast, Akt was not significantly altered. These findings showed that the crude methanol root bark extract of *Securinega virosa* possesses significant activity against GBM cells which may be mediated partly via EGFR and some other downstream mediators of cell survival.**

Key words: *Securinega virosa*, glioblastoma multiforme, epidermal growth factor receptor (EGFR), mitogen activated protein kinase (MAPK).

INTRODUCTION

Glioblastoma multiforme (GBM) is the most aggressive of the gliomas, a collection of tumors arising from glia or

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their precursors within the central nervous system. It accounts for 12 to 15% of all intracranial tumors and 50 to 60% of astrocytic tumors (Chang et al., 2014). It is characterized by features of vascular proliferation, hypercellularity, pleomorphism, and pseudopalisading necrosis (Huse and Holland, 2010; Ostrom et al., 2014). It is clinically classified into primary and secondary with the former accounting for more than 90% of all cases and arises as a *de novo* process (Chang et al., 2014). It has an average incidence rate of 3.19 in every 100,000 population, affecting more men and individual of white race and non-Hispanic ethnicity (Thakkar et al., 2014). Most patients with GBMs die of their disease in less than a year and essentially none has long-term survival (Holland et al., 2000). The standard treatment protocol for patients with this cancer involves surgical tumor resection followed by radiation with or without chemotherapy with alkylating agent, temozolamide (Stupp et al., 2005). Unfortunately, most of the treatment is palliative for most patients (Zahonero and Sánchez-Gómez, 2014). Sadly also, the use of this combination has not produced a significant improvement in GBM response rates, and the prognosis for GBM patients has remained dismal (Rao et al., 2003). The efficacy of these therapeutic approaches often is limited by non-specific toxicity due to non-exclusivity of their activity (Sampson et al., 2009). Epidermal growth factor receptors (EGFR) are involved in glioma formation, proliferation and invasion. EGFR has been reported to be amplified or over expressed in about 60% of GBM. It is a receptor tyrosine kinase which involved in cell proliferation, differentiation, and migration as well as apoptosis modulation (van der Geer et al., 1994). EGFR is of significant prognostic value in predicting survival (Shinojima et al., 2003) and upregulation has been found to be positively correlated with GBM malignancy while its signaling may be involved in resistance to irradiation therapy.

Traditional medicines have been used for ages for the management of human ailments including cancers (Corson and Crews, 2007). Medicinal plants constitute the cornerstone of traditional medicines. It is a fact that a number of anticancer agents in use today have their origin traced to medicinal plants (Shoeb, 2006; Balunas and Kinghorn, 2005). *Securinega virosa* is one of the great African medicinal plants described as a true "cure all", of which all parts are used as remedies, particularly the root. It is a dense, low branching, many branched shrub, sometimes a small spreading tree up to about 6 m high, although, more commonly 2 to 3 m, evergreen or deciduous (Neuwinger, 1996). The plant has enjoyed a wide patronage in traditional medicines for the management of a number of conditions including cancer (Tatematsu et al., 1991). The leaves of the plant are used in the management of cancer in the South Western Nigeria (Sholadoye et al., 2010). The decoction of the root plant and some other plants is used in North-Western Nigeria in the management of "*daji*", the most common term used to describe cancers by the Hausa speaking people of Northern Nigeria (Abubakar et al., 2007). The root extract of *S. virosa* has been reported to be active in bioassay against *Pyricularia oryzae* fungus, a preliminary screening of antineoplastic and antifungal agents (Tsuruo et al., 1986; Hu et al., 2001). This study, therefore, aims at evaluating the effect of the methanol root bark extract of *S. virosa* and its fractions on GBM cells survival.

MATERIALS AND METHODS

Collection of plant and preparation of extract and fractions

The plant material was collected in February, 2009, in Basawa town, Sabon Gari Local Government Area of Kaduna State, Nigeria and was identified by Umar Gallah of the herbarium section of the Department of Biological Sciences, Ahmadu Bello University, Zaria, Nigeria by comparing it with existing specimen (Number 918). A voucher specimen was subsequently deposited for future reference.

Extraction and fractionation

The root bark of the plant was removed, air dried and size-reduced. The powdered root bark of *S. virosa* (1000 g) was extracted with 4 L of absolute methanol using a Soxhlet apparatus over a period of 72 h. The resultant extract was concentrated *in vacuo,* yielding 9.5% of brownish residue subsequently referred to as crude methanol extract (CRE). The dried crude methanol extract (50 g) was dissolved in water and filtered. The filtrate was successively partitioned into chloroform and n-butanol. The fractions were separately concentrated *in vacuo* affording the following yields: chloroform (0.28 g), n-butanol (7.32 g) and residual aqueous (30.82 g) fractions. They were subsequently stored in desiccators before use.

Preliminary phytochemical screening

The CRE and fractions of *S. virosa* were screened for the presence of alkaloids, tannins, saponins, flavonoids and steroids/triterpenes using standard protocols (Silva et al., 1998).

Materials for anticancer study

Phorbol myristate acetate (PMA, 100 nM), EGF, PDGF and anti-tubulin antibody were purchased from Sigma Aldrich (USA). The phospho-specific antibodies directed against the EGFR at Tyr_{1068} and Tyr_{1045} and anti-EGFR antibody were purchased from Cell Signaling Technology, Inc. (Beverly, MA).

Cell cultures

The human U-1242 MG cell line was generously provided by Prof. Isa M. Hussani. U-1242 MG is one of the well characterized glioblastoma cell lines with mutant p53. The cell lines were originally isolated from astrocytic tumors that were designated as

Table 1. Preliminary phytochemical screening of the crude extract and fractions of *Securinega virosa*.

Constituent	CRE	CCF	NBF	RAF
Tannins	+	-	+	+
Saponins	+	-	+	+
Flavonoids	+	-	+	+
Alkaloids	+	+	+	+
Resins	+	+	+	+
Steroid/Terpenoids	+	+	-	+

+: Present; –: absent; CRE: crude methanol root bark extract; CCF: chloroform fraction; NBF: n-butanol fraction; RAF: Residual Aqueous fraction.

glioblastomas, and their characteristics have been described previously by Hussaini et al. (2000). Cell lines were regularly determined to be free of mycoplasma with reagent from Gen-Probe Inc. (San Diego). Cells were grown in modified α-minimal essential medium with 10% defined fetal bovine serum (Hyclone, Logan, UT) and 20 µg/ml bovine zinc insulin (25.7 IU/mg, Sigma). The cells were cultured to 100% confluence, passaged every 4 to 5 days from an initial concentration of 6 to 8 × 10^3 cm^{-2} in T flasks or 6- or 24-well plates, and cultured in astrocyte growth medium, 5% fetal bovine serum at 37°C in 5% CO_2 and 90% relative humidity. Prior to assays, cultures that were 80 to 100% confluent were washed three times with serum-free medium.

Cell survival assay

Cells in 6 well dishes were treated with dimethyl sulphoxide (DMSO), the crude methanol root bark extract or its fractions (CCF, NBF and RAF) at concentrations of 31.5 to 250 µg/ml; and incubated at 37°C for 48 h. Cells were subsequently washed three times with deionized water and stained with crystal violet dye. The cells were subsequently photographed. Adherent cells were released from the plate with 0.05% w/v trypsin-EDTA (0.53 mM) for 2 min. The trypsin activity was halted by addition of equal volume of serum-free α-MEM. 30 µl of the trypsinized cells was then collected in a cuvette and the viable cell count was determined using the O_2 coulter counter instrument (Beckman Coulter Inc.). The experiment was repeated three times and the values presented represent the mean number of cells.

In another study, cells were treated with DMSO, the CRE or its fractions (31.5 to 250 µg/ml). One hour later, EGF (25 ng/µl) was added to each plate. The cells were incubated for 48 h and the same procedures for cell count were carried out as described earlier.

Western blot analysis

Cells in 100 ml dishes were serum-starved for 24 h after reaching 80 to 100% confluence by replacing the medium with serum-free α-minimal essential medium. The cells were treated with the crude extract (CRE) and it fractions (CCF, NBF and RAF) at concentration of 250 ng/µl or DMSO (1 µl/ml) and allowed to stand for 1 h in the incubator. Thereafter, EGF (25 ng/ml), PMA (10 µM) or PDGF (20 ng/µl) were added separately and allowed to stand for 30 min in the incubator. After the different treatments, the cells were washed with phosphate-buffered saline (PBS; 137 mM NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCl, and 1.5 mM KH_2PO_4, pH 7.4) containing 0.2 mM sodium orthovanadate and extracted with 1% Triton X-100 (containing 2 mg/ml sodium orthovanadate and 5 mg/ml DTT) . Cells were centrifuged at 14,000 × *g* for 1 min at 4°C. The protein concentration of the supernatant was determined by the BCA protein assay (Bio-Rad). Proteins were boiled for 5 min in SDS-PAGE buffer. Proteins (200 µg/lane) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) polyacrylamide gels (8% for EGF and PMA; and 10% for PDGF) and then electroblotted onto nitrocellulose and reacted with mouse or rabbit antibodies specific for the EGFR, platelet derived growth factor (PDGF), mitogen activated protein kinase (MAPK) and Akt. Immunoblotted proteins were subsequently detected using the ECL reagents as described by the manufacturer with horseradish peroxidase-conjugated secondary antibodies (Hussaini et al., 2000).

Statistical analysis

The difference between the control and the test groups were analyzed for statistical difference using one way analysis of variance (ANOVA) followed by Dunnett post hoc test for multiple comparisons. P values less than 0.05 were considered significant.

RESULTS

Preliminary phytochemical screening

The preliminary phytochemical screening revealed that the residual aqueous fraction contained similar constituents with the CRE. The chloroform fraction was deficient in tannins, saponins and flavonoids (Table 1).

Cell survival assay

Treatment of the cells with EGF resulted in 38% increase in cell count compared with DMSO treated cells (Figure 2). The crude methanol extract (CRE) and its fractions significantly ($P < 0.001$) and concentration- dependently

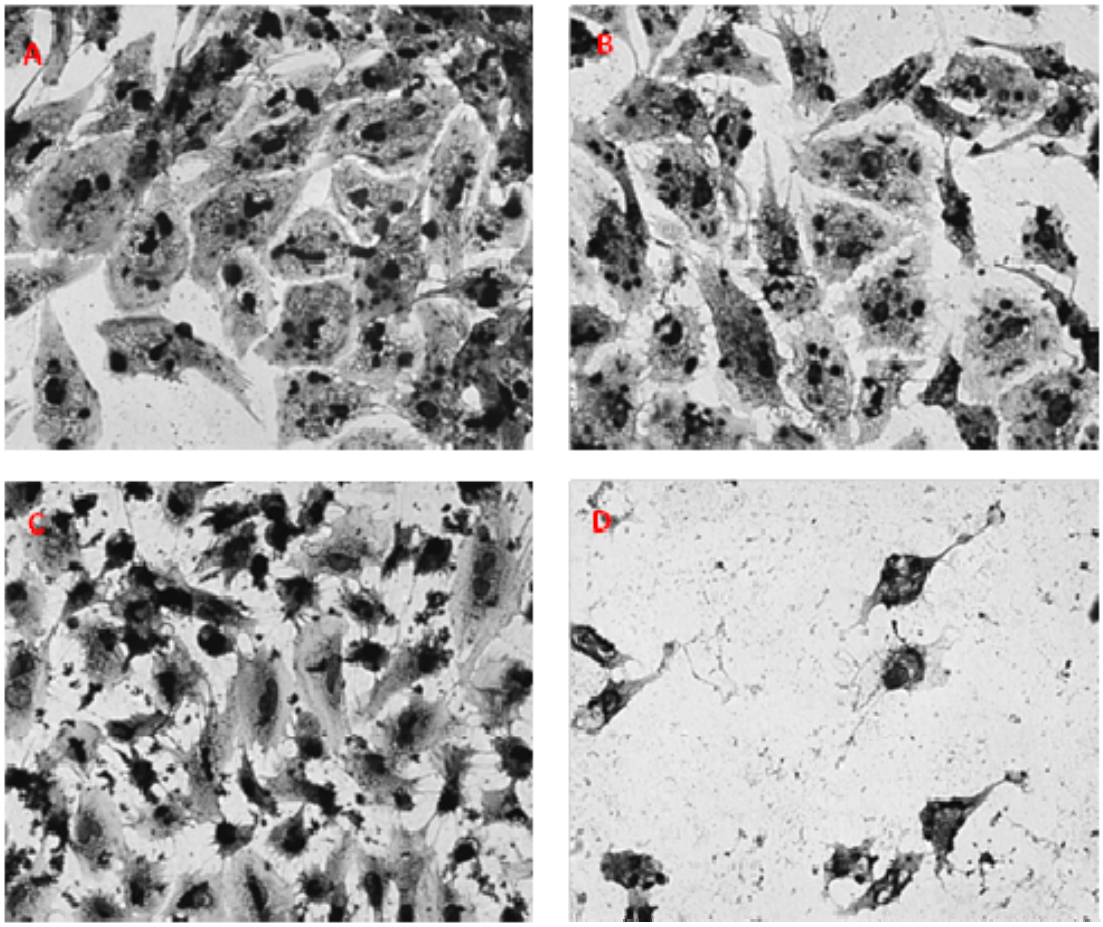

Plate 1. GBM U-1242 MG cells treated with either DMSO or the crude extract. After 48 h cells were stained with crystal violet dye and observed under microscope (×20) A: DMSO; B: CRE 62.5 µg/ml; C: CRE 125 µg/ml; D: CRE 250 µg/ml.

Table 2. IC_{50} values of the Crude methanol root bark extract of *Securinega virosa* and its fractions against Glioblastoma muliforme cells

Extract/Fraction	IC_{50} value (µg/ml)
CRE	41.035
RAF	29.809
NBF	58.502
CCF	4.872

CRE: Crude methanol root bark extract; CCF: chloroform fraction; NBF: n-butanol fraction; RAF: residual aqueous fraction.

decreased cell viability of the U-1242 MG cells in the presence and absence of EGF (Plate 1, Figures 1 and 2). The highest activity was observed with the chloroform fraction with an IC_{50} value of 4.87 µg/ml (Table 2).

Western blot analysis

The crude methanol root bark extract (CRE) of *S. virosa* blocked the phosphorylation of EGFR at tyr 1068 site. It

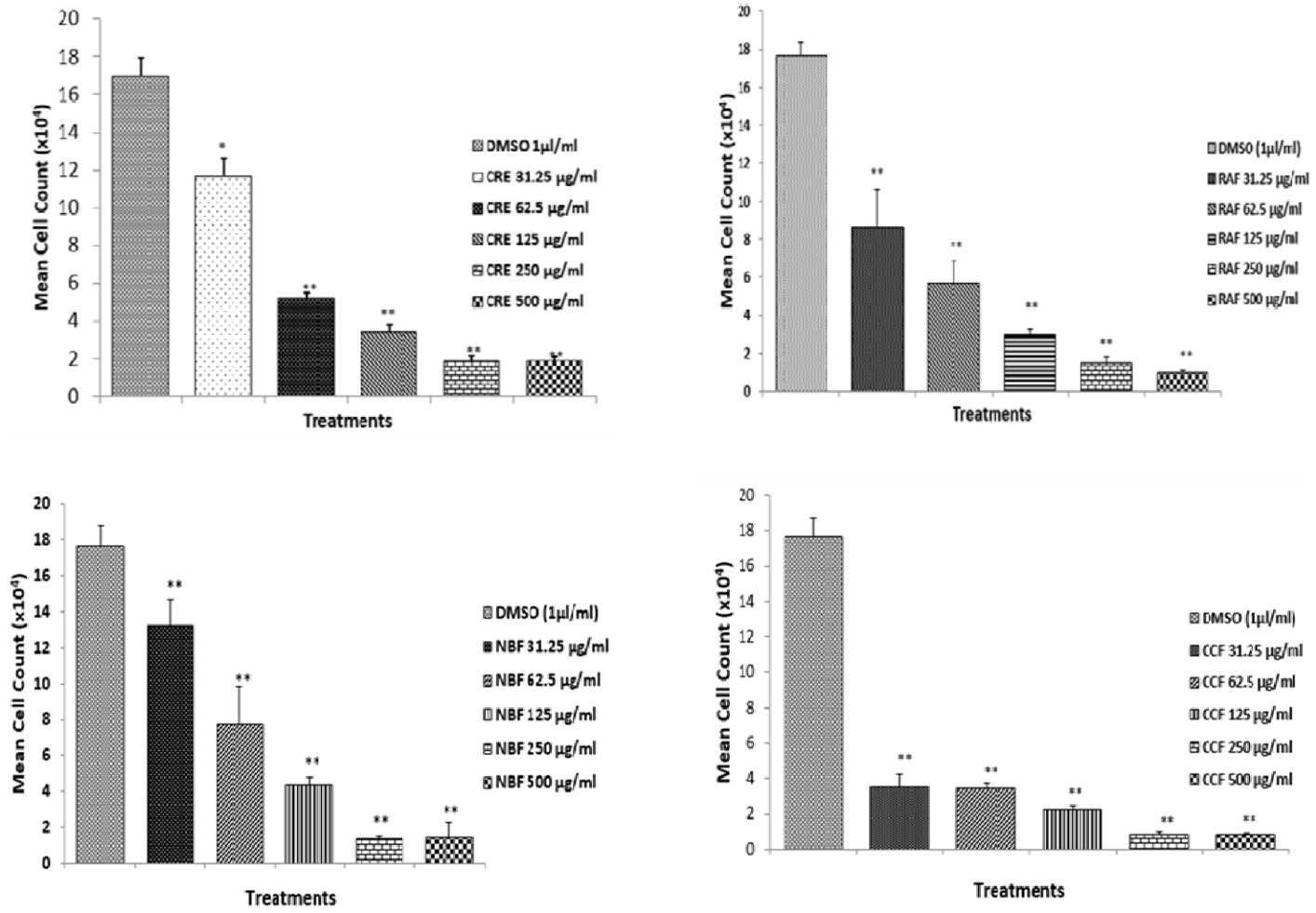

Figure 1. The effect of crude methanol root bark extract of *Securinega virosa* and its fractions on cell survival of GBM cells U-1242 MG. DMSO: Dimethyl suphoxide; CRE: crude methanol root bark extract; RAF: residual aqueous fraction; CCF: chloroform fraction. Data presented as mean ± SEM; *P < 0.05; **P < 0.001; n=4.

also blocked the phosphorylation of MAPK (Figure 3). The CRE, residual aqueous fraction and n-butanol fraction blocked the phosphorylation of EGFR at tyr 1045. However, the activity was more remarkable with the CRE and residual aqueous fraction. Only the crude extract and the residual aqueous fraction remarkably blocked EGFR phosphorylation at Tyr 1068 site (Figure 4). The CRE significantly blocked PMA-induced transactivation of EGFR at Tyr 1068 site. The effects produced by the residual aqueous and n-butanol fractions were less remarkable (Figure 5). The CRE significantly blocked the phosphorylation of platelet derived growth factor receptor (PDGFR). The extract and the residual aqueous fraction remarkably blocked PDGF-induced transactivation of EGFR at Tyr1068. Conversely, the extract as well as its fractions produced no effect on the phosphorylation of Akt (Figure 6).

DISCUSSION

In the present study, the effect of the methanol root bark extract of *S. virosa* and its fractions on GBM cell survival was evaluated. The extract and its fractions possess significant activity against the GBM cells viability in the presence of EGF. GBM is the most common and aggressive primary CNS tumor with a median survival of 15 months and an average incidence rate of 3.19 in every 100,000 population (Thakkar et al., 2014). GBM cells are highly proliferative and diffusely invade surrounding brain

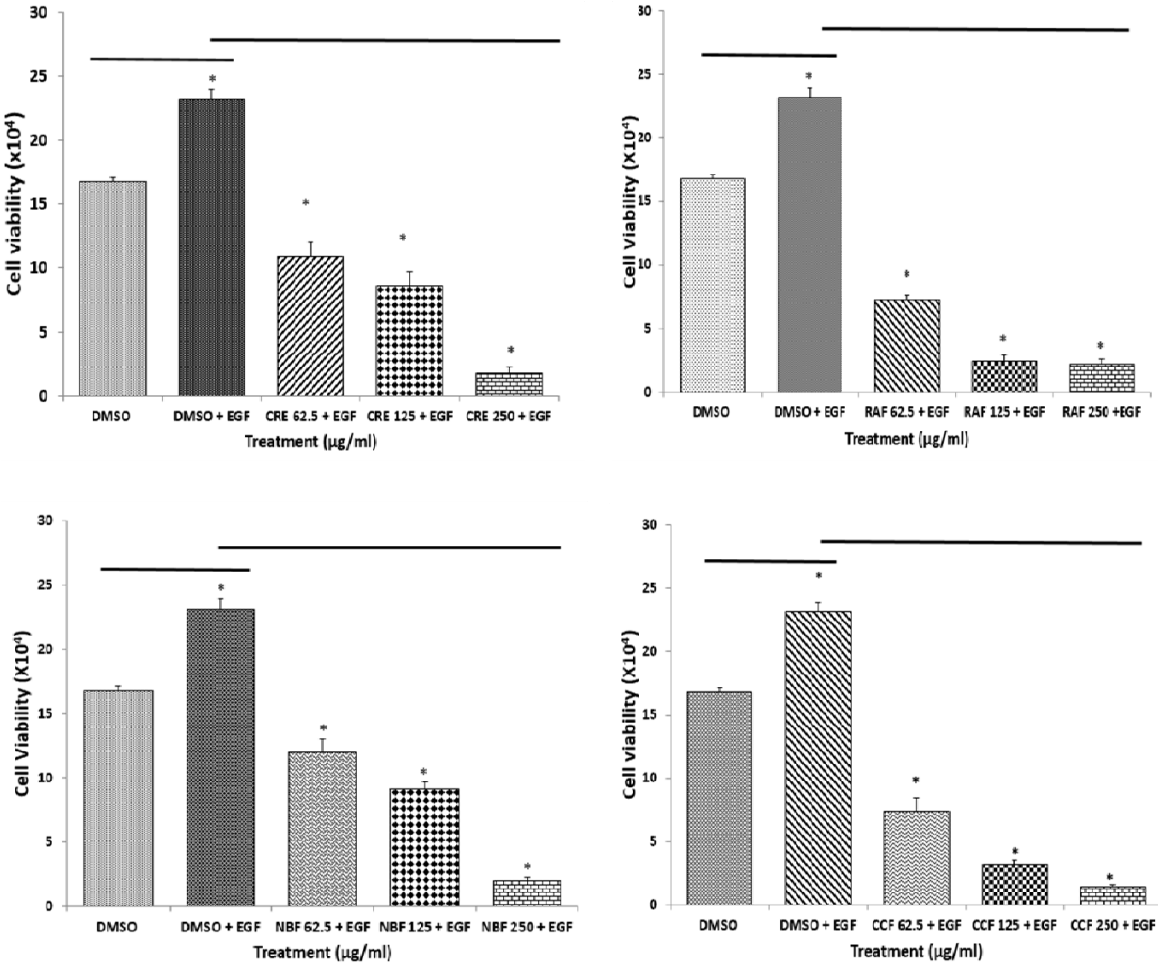

Figure 2. The effect of crude root bark extract of *Securinega virosa* and its fraction on viability of U-1242 MG GBM cells treated with epidermal growth factor. Cell count was carried out 48 h after the treatment. DMSO: Dimethyl sulphoxide; EGF: epidermal growth factor; CRE: crude methanol root bark extract; RAF: residual aqueous fraction; NBF: n-Butanol fraction; CCF: chloroform fraction. Data presented as mean ± SEM; *P < 0.001; n=4.

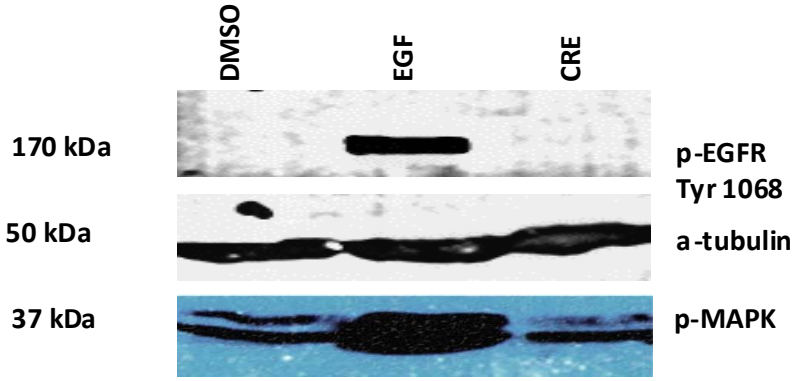

Figure 3. The effect of methanol root bark extract (CRE) of Securinega virosa on phosphorylation of EGFR receptor at tyr 1068 site and mitogen protein kinase (MAPK).

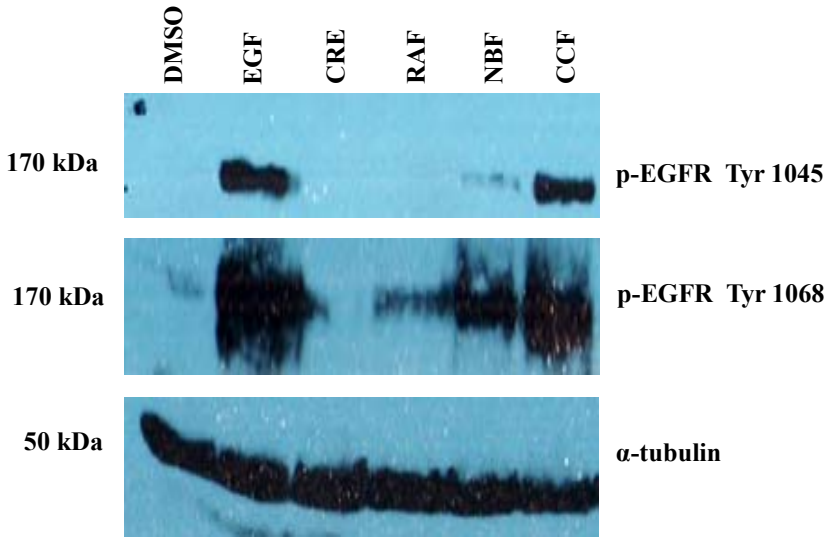

Figure 4. Effect of crude methanol root bark extract of *Securinega virosa* and its fractions on EGF induced phosphorylation of EGFR at tyr 1045 and 1068 sites.

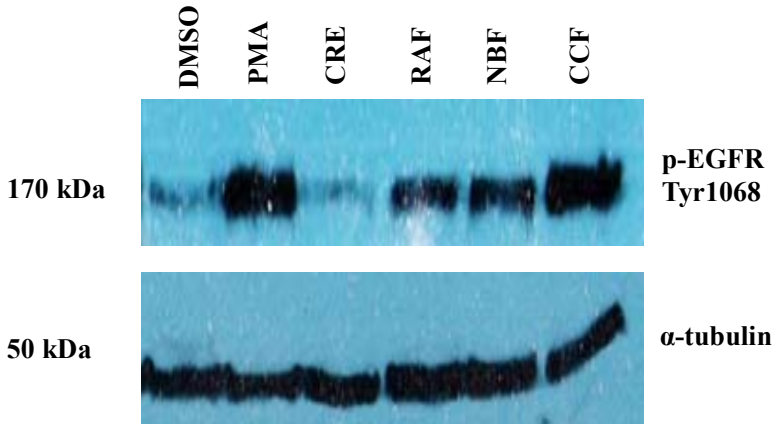

Figure 5. Effect of methanol root bark extract of *Securinega virosa* and its fractions on PMA- induced transactivation of EGFR at tyr 1068 site.

structures, thereby making complete surgical resection practically impossible (Barcellos-Hoff et al., 2009). Furthermore, majority of GBMs are intrinsically resistant to most forms of radiation and chemotherapy, thus rendering the standard arsenal of anticancer treatmentsrather ineffective (Reardon and Wen, 2006). The failure of the conventional therapies for GBM to target tumor cells exclusively, make their efficacy limited by non-specific toxicities (Sampson et al., 2009). Therefore, killing tumor cells efficiently and with less toxic effect is the ultimate target of GBM chemotherapy and will require the adoption of targeted therapeutic approach as against the deployment of cytotoxic arsenal (Zahonero and Sánchez-Gómez 2014).

GBMs commonly overexpress oncogenes such as EGFR and PDGFR and contain mutations and deletions of tumor suppressor genes such as phosphatase and tensin homolog (PTEN) and tumour suppressor protein 53 (TP53), all of which can have an impact on the activation state of signal transduction pathways that influence their biological behaviour (Mischel and Cloughesy, 2003). EGFR is a tyrosine kinase receptor which is over-expressed in cancer cells, and has been identified as a target of tumor therapy. 50 to 60% and

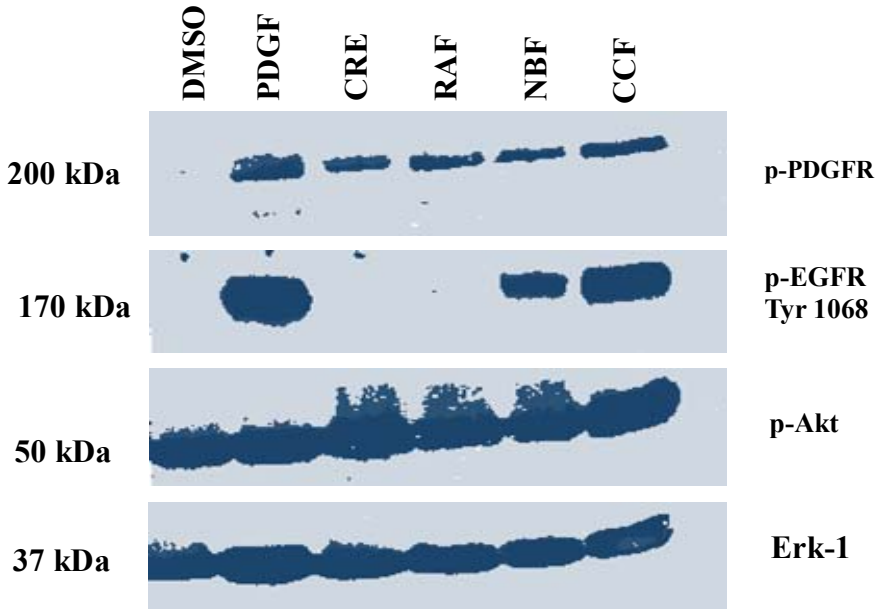

Figure 6. Effect of methanol root bark extract of *Securinega virosa* and its fraction on PDFR-induced phosphorylation of PDGFR, transactivation of EGFR at tyr 1068 receptor site and phosphorylation of Akt.

40% of GBMs have overexpression and amplification of the EGFR, respectively (Ohgaki et al., 1995). The EGFR or ErbB family belongs to subclass I of the superfamily of the receptor tyrosine kinases. Receptor tyrosine kinases represent an important subclass of these transmembrane proteins, with the EGFR being the most prominent representative. The EGFR controls a wide variety of biological processes such as cell proliferation, differentiation, and migration and modulation of apoptosis (van der Geer et al., 1994).

The EGFR variant III is the most commonly occurring mutation of the EGFR in the glioblastoma and is present in 25 to 33% of all GBM (specifically in those showing EGFR receptor amplification and overexpression) (Aldape et al., 2004). This variant is a product of the deletion of exons 2 to 7 and consists of an in frame deletion of 801 bp from the extracellular domain of the EGFR that splits a codon and produces a novel glycine at the fusion junction, rendering the mutant receptor incapable of binding any known ligand (Gan et al., 2013). The mutation encodes a constitutively active tyrosine kinase which enhances tumorigenic, cell migration and confers resistance to radiation and chemotherapy (Chu et al., 1997; Sampson et al., 2009). The absence of this mutation in normal tissues makes it a potential target for tumor specific therapy (Pelloski et al., 2007). Therefore, concerted efforts are been channeled towards the development of anti-EGFR III agents including strategy such as immunotherapy and antibody neutralization (Reardon et al., 2014).

The methanol root bark extract and its residual aqueous fraction blocked the phosphorylation of the EGFR at tyrosine 1045 and 1068 sites. This suggests the involvement of these sites in the activities of the extracts. The blockage of PDGFR phosphorylation is an indication that the activity of the extract may not be limited to EGFR. Similarly, PMA induced transactivation of EGFR at tyr 1068 were also blocked. PMA has been reported to phosphorylate the EGFR at Tyr1068 through a PKC/c-Src-dependent pathway resulting in the activation of MAPK and increase in cell proliferation in glioblastoma cells (Amos et al., 2005).

MAPK phosphorylation was also blocked by the crude methanol root bark extract. The blockade of MAPK phosphorylation by the crude methanol extract suggests that its activity may involve some downstream mediators. However, the extract and its fractions did not affect the phosphorylation of Akt suggesting that their activity may not involve PI3k/Akt pathway.

Although the chloroform fraction gave the highest activity in terms of reduction in cell viability with an IC_{50} of 4.87 µg/ml, its mechanism of action may not involve EGFR, PDGFR, MAPK or Akt; but may be a necrotic phenomenon.

The ability of the extract and fractions to block the phosphorylation of EGFR and PDGFR suggests that they

may be acting via tyrosine kinase receptors. However, there more pronounced activity on EGFR, there blockade of PMA and PDGFR mediated transactivation of EGFR at Tyr1068 suggest that they may be more specific for the EGFR.

Alkaloids, tannins, saponins and flavonoids have been reported to possess anticancer activities (Stevigny et al., 2005; Miyamoto et al., 2003; Kaskiw et al., 2009; Ren et al., 2003). Virosecurinine and viroalloscurinine alkaloids isolated from the leaves of *S. virosa* have been shown to possess some cytotoxic activity (Tatematsu et al., 1991). Interestingly, ethyl acetate fraction which was found to be deficient in alkaloids did not show significant anti-GBM activity (data not shown), suggesting that alkaloids may be responsible for the observed activity. It is therefore plausible to suggest that these phytochemical constituents found to be present in the CRE and fractions of *S. virosa* may be responsible for the anticancer potential of the root of *S. virosa*.

The findings of this study lend some credence to the ethnomedical use of the root of the *S. virosa* in the management of cancer. Further work will involve toxicity evaluation of the extract and its fractions in non-cancerous cell lines as well as isolation of the bioactive principle(s) responsible for these activities, particularly, the alkaloids which have been previously documented to exhibit anticancer property.

Conflict of Interest

The authors have not declared any conflict of interest.

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