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The Effects of Zizyphus Stem Bark and Termite Shelter Tubes on HeLa cell Growth.

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ABSTRACT

This study tested the effect of ethanolic extracts of Zizyphus stem bark and termite shelter tubes on HeLa cell line in small scale culture in vitro. The growth of HeLa cells was investigated at three different concentrations of each extract. Cells were cultured in 96 wells plate treated for tissue culture, the cells were incubated for 24 hours then proliferation was assessed and compared to untreated cells. The treated cells showed a significant increase in proliferation rate for all extract concentrations. These results were augmented by the significant increase in glucose consumption.

Keywords: HeLa, Zizyphus, cell growth.

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INTRODUCTION

Treatment of a wound should aim to promote healing which is a continuous process that can be divided into four phases, of which cell proliferation (third phase), which starts within days of the injury, encompasses the major healing process (Velnar *et al*., 2009).

Natural products have been traditionally used to promote wound healing. Researches on some of these products provided a scientific base for their use (Maenthaisong *et al*., 2007, Srivastava *et al*., 2010). Many of these products have pharmacological or biological activity that can be utilized in drug discovery (Newman *et al*., 2003, Butler, 2004). A number of drugs synthesized from natural product are actually produced by microbes or microbial interactions with host (Newman & Cragg, 2007).

Some of the local population in Iraq have been using a powder mixture of zizyphus stem bark and shelter tubes of termites (infesting date palm trees) to treat skin burns, apparently to avoid the painful process of regular scrapping and washing routinely used in modern burn therapy.

Zizyphus stem bark

Zizyphus spina-christi locally known in Iraq as Nabaq or Sidr is a tree species belonging to the genus Zizyphus of the botanical family Rhamnaceae. Many parts of the tree has been used for different purposes in folklore medicine (Mayssaa *et al*., 2011). Leafs, studied to explore their biological effects, have shown concentration dependent cytotoxic activity (Jafarian *et al*., 2014). Stem park of the tree is light-grey, very cracked, and scaly (Haghighat, 2012). The ethanolic extract of the bark contain several phytochemical compounds including carbohydrate, Soluble starch, tannins, flavonoids, terpernoids, alkaloids, saponins, and cardiac glycosides(Mohammed *et al*., 2013). The bark has shown to have an antibacterial and antifungal activity attributed to its phytochemical content (Yesufu *et al*., 2012, Mohammed *et al*., 2013, I. S. Sadiq, 2013). Although other species of Ziziphus has shown positive effect on cell growth (Hwang *et al*., 2011), and wound healing (kumar *et al*., 2012), there were limited resources regarding that of Zizyphus spina- Christi.

Termite shelter tubes

Termites are members of the order Isoptera, comprising roughly over 2600 species (Culliney, 2013).The small waxy termite species *Microcerotermes diversus*is is known to infest date palm trees (*Phoenix* dactylifera) in Iraq. They can be noticed on the surface of the infested tree by their characteristic mud tunnels (Shefik, 2010). In their seek for food, Foraging termites build shelter tubes on the trunk and basal leaves of the infested tree (Kaakeh, 2011), they feed mainly on wood as they contain cellulose-degrading bacteria in their gut (Pourramezan *et al*., 2012). Walls of their tubes are built from a mixture of clay, saliva and feces (DeSouza & Cancello, 2011). Lignin is the main constituent of fecal pellets with small amount of undigested cellulose (Lavelle *et al*., 1997). Lignin has shown anti-proliferative effect on both normal and cancer cells (Andrijevic *et al*., 2007,Iio & Yamafuji, 1968), while cellulose was found to have positive effect on bone cell growth (De Taillac *et al*., 2004) and was successfully used as a scaffold to support cell proliferation and adhesion in vitro (Favi *et al*., 2013), it is unfortunately do not dissolve in ethanol (Horvath, 2006) . In addition, Termites secrete strong antimicrobial compounds in faecal pellets or body secretions (Grace, 2003, Sujada *et al*., 2014).

Healing of skin burn wounds remains a challenging clinical problem. Studying the effects of various experimental products on *in vitro* cell growth can pave the way for further studies *in vivo*. Powders of Stem Park of Zizyphus spina-christi and shelter tubes of the termite (*Microcerotermes diversus*) and their mixture are used in this study to assess their effect on HeLa cell line proliferation. There have been no studies concerning this mixture.

MATERIALS AND METHODS

Sample collection

The stem bark of zizyphus spina-christi and shelter tubes of termites (*Microcerotermes diversus*is) was collected from trees in the local community. They were of an easily excavatable size and were wrapped in black plastic bags and labeled for up to 24 h in the laboratory.

Extracts Preparation

After cleaned, the stem bark samples were dried in the oven at 40 Cº for 72 hours, then milled and kept refrigerated in plastic bags for future use. Ethanolic extracts were prepared according to (Shinde & Gawai 2011), by dissolving 20gm of the bark powder in 10ml of 96% ethanol and left for 24hrs at room temperature, then filtrated using Whatman No.1filter paper. The extract then dried in the oven at 40 Cº for 3-4 days. The required concentration of the extract was prepared by dissolving a suitable weight of the extract powder in a suitable volume of PBS in order to obtain the stock concentration which finally sterilized by 0.22 µm Millipore filter. The shelter tubes of termites extract was prepared in the same way as mentioned above.

Cell line

HeLa cell line **(**cervical cancer**)** were seeded in 96-wellplates and incubated in the presence of different doses of extracts in order to detect the existence of a dose response and its effects. The plant concentrations used were (100, 200 and 300µg/ml) respectively. The plated cells were divided into four groups:

Group1 was the control group (not treated); group2 received 100µg/ml final concentration of the extracts; group3 received 200µg/ml final concentration of the extracts; and group4 received 300µg/ml final concentration of the extracts. The cells were incubated at 37°C in CO_2 incubator for 24 hours before counting then the supernatants were collected and the cells were detached for the estimation of glucose concentration and cell counting respectively.

Glucose Concentration

The glucose concentration was determined with Ascencia Microfill test strips on Ascencia Counter Glucose meter. The Microfill strip was inserted into the device, one drop of each sample was placed on the tip of the test strip using 20µl pipette. The sample is taken up by the strip through capillary action. After 10 seconds the glucose level was indicated on the display in mg/ml or mM (Kuystermans & Al-Rubeai, 2009).

Trypan Blue Exclusion Method for Cell Counting and Viability Assessment

Viable cell number and viability were assessed by cell counting in an improved Neubaure hemocytometer under phase contrast microscope**.** One hundred microliter of cells were diluted with the same volume of trypan blue, mixed well by pipette and transferred on to both counting chambers of hemocytometer and counted. Trypan blue exclusion was also used to estimate the viability percentages of the cells (Strober, 2001).

RESULTS

Effects of Zizyphus spina- christi on HeLa cell proliferation

HeLa cells proliferation rate was significantly elevated after 24 hours incubation with Zizyphus extract at all concentrations compared to control group. The concentration 100µg/ml of plant extracts yield a significantly better effect on cell growth (Fig. 1). These results were supported by the glucose concentrations of the culture media that indicated significant depletion of glucose from the culture media due to increased proliferation rate (Fig. 2).

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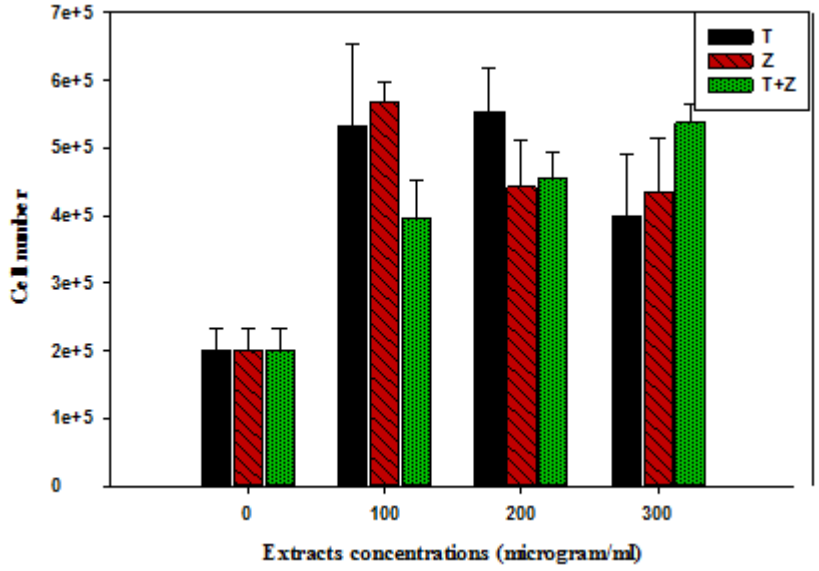

Figure 1: HeLa cells proliferation rates after 24 hours incubation with different concentrations of termite shelter tube extract (T) and Zizyphus extract (Z) separated or in combinations (T+Z)

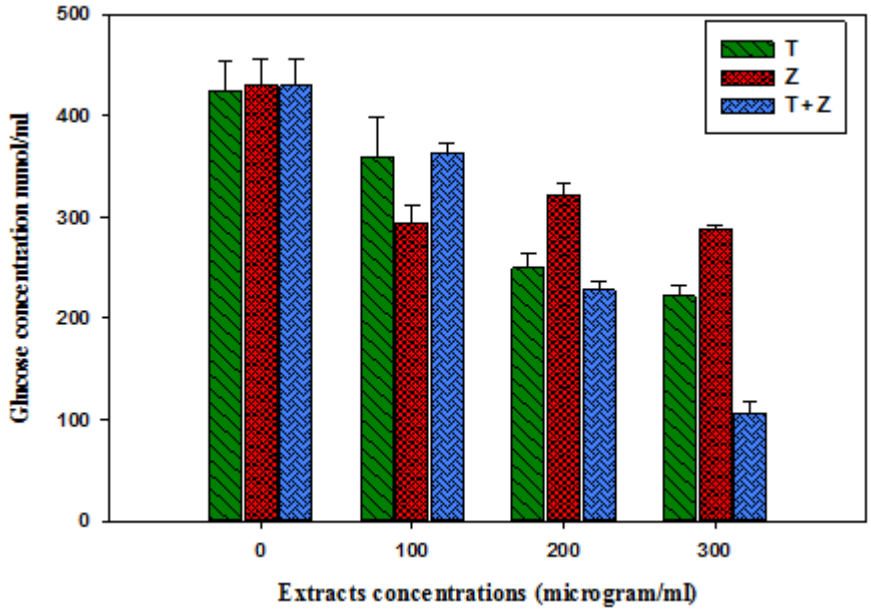

Figure 2: Glucose levels in culture media after 24 hours incubation with different concentrations of termite shelter tube extract (T) and Zizyphus extract (Z) separated or in combinations (T+Z)

Effect of termite shelter tubes on HeLa cell proliferation

As with zizyphus extract, Viable HeLa cells counted 24 hours after incubation with termite shelter tubes extract showed significantly higher rates of proliferation at all concentration levels compared to control group (Fig. 1). There was no significant difference between concentrations. These results were supported by the glucose concentrations of the culture media (Fig. 2).

Effect of Zizyphus and termite shelter tube mixture on HeLa cell line proliferation

HeLa cells proliferation rate was significantly elevated after 24 hours incubation with a 1:1 mixture of Zizyphus and shelter tube extracts at all concentrations compared to control group. (Fig. 1) In addition the concentration 100µg/ml of each extract yields a significantly better effect on cell growth. In contrast, mixture

of 300µg/ml of both extracts yields better effect on cells growth. Rapid depletion in glucose concentrations of the culture media supported these results (Fig. 2).

DISCUSSION

Previous studies on leaves extract of Zizyphus spina-christi have shown concentration dependent cytotoxic activity that increases at higher concentrations (Jafarian et al., 2014). In the present study, the extract of zizyphus stem bark have shown a concentration dependent growth promoting potential with a concentration of 100 µg/ml being significantly better than higher concentrations. This suggests the need for further studies to explore the concentration spectrum for the best concentration that can augment cell growth. One possible assumption to explain the growth enhancement is that zizyphus spina-christi stem bark extract has been shown to have antioxidant activity. Phytochemical analysis for the ethanolic extract showed the presence of cardiac glycosides, saponins, Flavonoids, alkaloids and tannins. These active compounds act singly or in synergy to give the observed antioxidant activity of these plant species (Asgarpanah & Haghighat, 2012, Abalaka et al. 2011),(Mohammed et al .2013). Antioxidant activity can modulate cellular growth via regulating the reactive oxygen species (ROS) levels inside the cells which function as second messengers in the control of cell proliferation. In this way, free radical scavenging potential activates cell cycle and increases cell proliferation. (Sauer et al,2001, Fartes et al, 2012, Ebadi, 2007).

Ethanolic extract of termite shelter tubes activated cell proliferation with less variation at different concentrations. As mentioned above, fecal pellets sharing the formation of termite shelter tubes are formed mainly from lignin with cellulose to a lesser degree. The cellulose has a complex structure and does not dissolve in ethanolic extracts, lignin, on the other hand is known to dissolve (Horvath, 2006). These results came in contrast to (Iio and Yamafuji, 1968) and to (Andrijevic et al., 2007). This can be attributed to the difference in the composition of lignin used in these studies. A study comparing the effects of synthetic to natural lignin is here by advised. In addition, a recent study carried out in the University of Florida discovered that faecal nests also support the growth of Actinobacteria (Chouvenc et al, 2013) suggesting the presence of some nourishing digested materials excreted within the feces of this insect. Palm tree itself contain compounds similar to that found in zizyphus stem bark and thus palm extract has an antioxidant activity (Eddine et al, 2014), we can suggest here that some of these compounds are retained in the feces giving this growth enhancement activity. Unfortunately, the composition of the termite shelter tubes have not been fully explored yet, further studies to unveil its chemical constituents.

The mixture of the two extracts gives excellent results at higher concentration. These results suggest the presence of a type of interaction between the components of these two extracts that modulate its effect. Further studies to explore wider spectrum of concentrations and experimental trials to investigate its effects on animal models are suggested to explore the healing potential of this mixture.

Whereas all these results explains the basis of the use of the mixture in the treatment of skin burns, fractions of the mixture are worth probing for development of new drug.

CONCLUSIONS

At certain concentrations the mixture and its individual components have positive effects on cell growth. The mixture under study is therefore may have a scientific base to support its use in folk medicine for burn treatment. Studies to isolate the active ingredients of the extract that promote cell proliferation and growth are recommended before proposing its potential application for therapeutic use.

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