Effect of Combination of Pure Isolates of *Eichhornia Crassipes*' Roots on Bioactivity Against Some Cancer Cell Lines

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Abstract. The fight against cancers still demands robust research from every possible angle including phytomedicine to find safer and more effective agents or approaches to therapry. One such approach is combinatory therapy. This work aimed at isolating active compounds from *Eichhornia crassipes'* roots that would be tested for activity against MCF-7 and HepG2 cell lines both individually and in combination. UV Chromatograms from a liquid chromatography with a Mass Spectroscopy detector was used to confirm the purity of the isolated compounds. MCF-7 and HepG2 cell lines were subjected to four compounds (1-4) isolated from the roots of *Eishhornia crassipes* with doxorubicin as a standard. Compound 3 was the most active against MCF-7 with an IC_{50} of less than 4 ug/ml. When combined in pairs, a combinatory dose of compounds 2 and 3 was the most active against HepG2 (IC_{50} = 3.1 ± 0.16 ug/ml) while that of compound 3 and 4 had the highest activity against MCF-7 with an IC_{50} of 3.82 ± 0.06 ug/ml. It was noted that not every combinatory dose has an additive effect. Only 40.6% of the tests showed increased activity, 34.4% showed reduced activity and there was no change in activity in 25% of the tests. Combinatory doses require logical strategies for better clinical outcomes.

Keywords: Combination therapy, Drug Resistance, *Eichhornia crassipes*, Synergy, Antagonistic effect, Purified compounds

Introduction

Cancer remains a huge burden in the world whose drugs are hard to access due to exhorbitant costs (Arbyn et al., 2020; Biemar & Foti, 2013; Siegel, Miller, & Jemal, 2020). Developing new drugs against cancers takes a very long time and is very costly (Bayat Mokhtari et al., 2017; Bian & Xie, 2020; Lobo, 2020; Xu et al., 2020). Apart from costs, cancer is fast becoming resistant to most drugs on the market (Cao et al., 2020; Eguchi et al., 2020; Narayanan et al., 2020; Wang et al., 2020). In view of this, researchers are still working on various discovery projects to find a very effective and safe cure for cancer that can also be accessible to the poor.

Plants are still providing compounds that are being explored for potential sources of leads against cancers. *Eishhornia crassipes* is one such plants that has been reported to contain some active compounds against some cancers (Mtewa, Sesaazi, & Lampiao, 2020). As research continues to find a cure, various anticancer drugs on the markert have been administered in combination to potentially enhance the activity of the drugs.

Combinatory dosing is one of the most common modes of drug administration where two or more drug regimens are adminsitered to patients against a single disease. It is one of the modalities that is pursued in cancer therapy taking advantage of its characteristic additive and syngergistic effects (Bayat Mokhtari et al., 2017; Derakhshani et al., 2020; Fares et al., 2020; Garjón et al., 2020). This approach has been proven to have several benefits in cancer and disease management in general. Apart from providing better tumor growth reduction, inducing mitotic cell arrests and reducing on the potential of proliferation, combinatory dosing also has

the potential to minimize drug resistance (Bayat Mokhtari et al., 2017; Coates et al., 2020). Already exisiting drugs are being repurposed and combined with other drugs for such better results.

There are already over 10,000 studies that are ongoing and were registered in the United States alone that are exploring combinatory therapy against various diseases (Editorial, 2017). Since much focus is on already existing drugs, efforts also need to be extended to the exploration of combinatory dosing for natural products which are currently being explored almost world wide. The current work aimed at isolating compounds from *Eichhornia crassipes* roots, determine their acitivity against breast cancer cell lines (MCF-7) and hepatocellular cancer cell lines (HepG2) individually and then in combination with each other. Combinatory doses are considered as successful if they produce better activity than individual drugs or agent for a particular disease (Coates et al., 2020; Editorial, 2017).

Methods

Sample Collections and Preparation

Ethyl acetate and n-hexane were purchased from Sigma-Aldrich (Germany) and LCMS from Agilent technologies (California, USA). The roots of *Eichhornia crassipes* were collected from Shire river in Malawi at 15˚03'11.3''S 35˚31'12.5''E in February, 2019 and was identified by Mr. Hassam Patel of the Malawi Herbarium and Botanic Gardens where the plant can be accessed with accession number 35964. The roots were washed using tap water and then rinsed several times using distilled water. The roots were dried and milled to fine powder and stored at -10 degree celcius for subsequent tests.

Isolation and Mass Identification of the Compounds

The powder was marcerated in methanol (Oroian, Dranca, & Ursachi, 2020) for 24 hours followed by gradient separation using n-hexane:Ethyl acetate in the following ratios: 90:10, 80:20 and 75:25 and then purified using column chromatography. The purified compounds were dried completely using a rotor vaporator (Buchi) and stored at -10^{o} C. The purity of the compounds was verified using a Liquid Chromatography (Agilent) complemented with a Mass Spectrometry detector which provided the masses of the isolated compounds.

Cytotoxicity Assays

Breast cancer cell lines (MCF7) and Human hepatocellular cancer cell lines (HepG2) cell lines and analysed as done by Aboul-Elneine and others (Aboul-Enein et al., 2014). Cell lines were prepared and stored in Roswell Park Memorial Institute (RPMI) 1640 culturing medium where there was penicilion (100 units/ml, 100 µg/ml of streptomycin and 10% fetal bovine serum) at 37˚C in a 5% CO_2 environment. 0.25% Trypsin-EDTA was used to collect the cells then they were plated in a 96-well plate in the range of 1.0 x10^3 to 2.0 x 10^3 cells/well. Doxorubicin (Sigma Aldrich) as a standard and isolated compound were transferred to the wells separately and incubated for 72 hrs. For combinatory dosing test, the combinations of the isolates were done in pairs at a 1:1 ratio, all under room temperature and the same conditions. Trichloroacetic acid (10%) was added to the wells at 4˚C and the wells were let stand for 1h. The cells were later washed then added to 4% of dye in dark for 10 minutes then washed with 1% acetic acid (glacial). The cells were left to dry overnight followed by the addition of Tris-HCl to dissolve the cells that were by now stained with the SRB dye. A PerkinElmer Victor X3 Multimode plate reader at 540nm was used to read the dye intensity. Graphpad Prism was used to calculate IC_{50} values.

Results and Discussion

Four isolates were obtained and they were pure as evident from the chromatograms (Figures 1 to 4) with almost a flat baseline, good enough for natural products. The weights of the compounds were as shown in Table 1.

Table 1. Weight of the isolated compound from the roots of *Eichhornia crassipes*

Compound	Weight (mg)
1	7.7
2	16
3	9.2
4	11

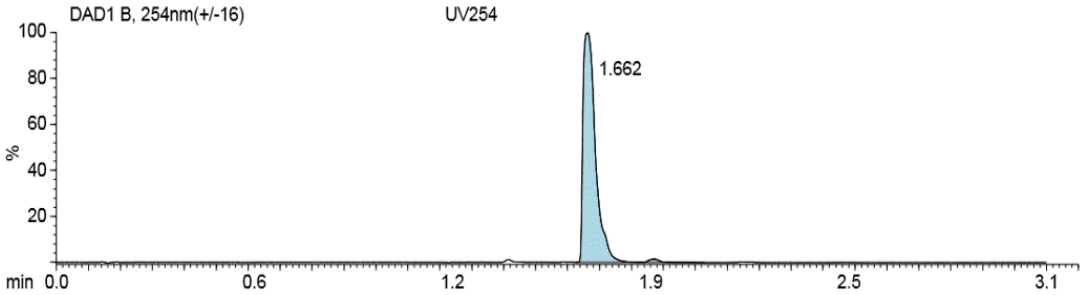

Figure 1. Chromatogram of isolated Compound 1

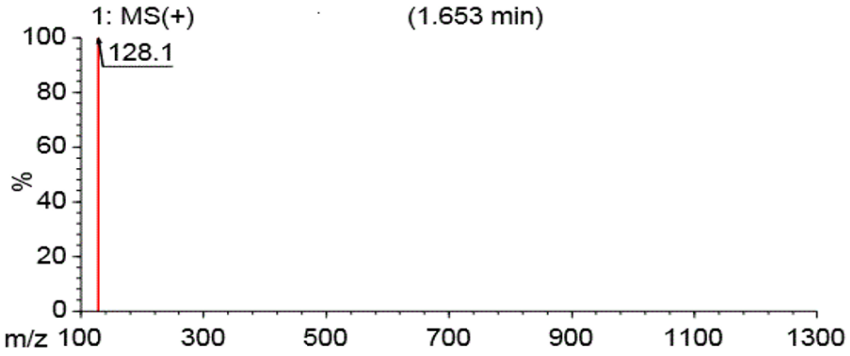

Figure 2. Mass spectral data for Compound 1

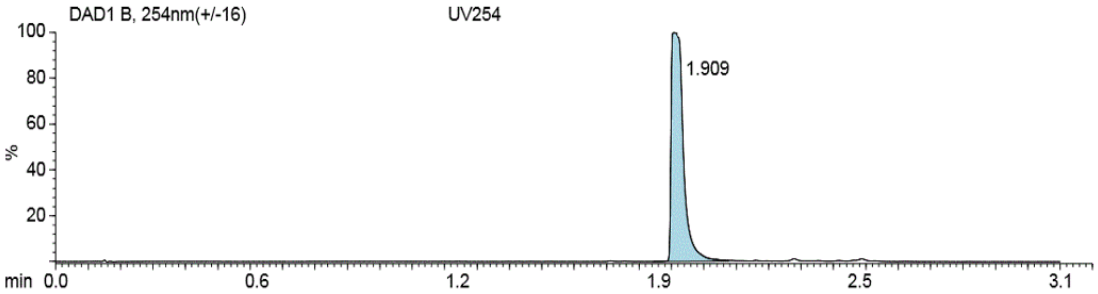

Figure 3. Chromatogram of isolated Compound 2

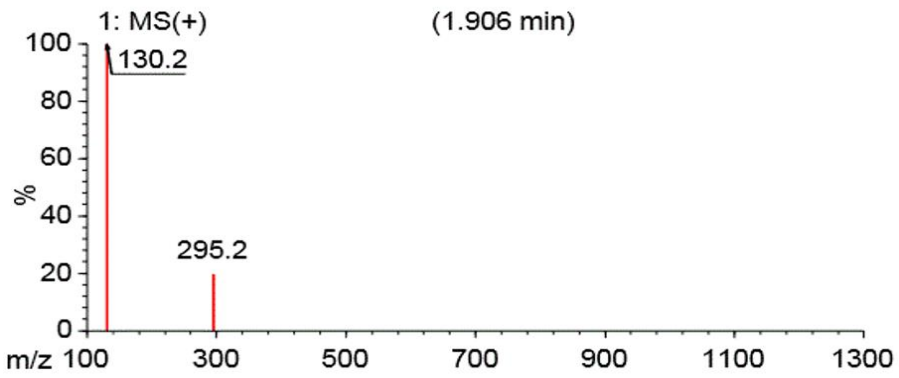

Figure 4. Mass spectral data for Compound 2

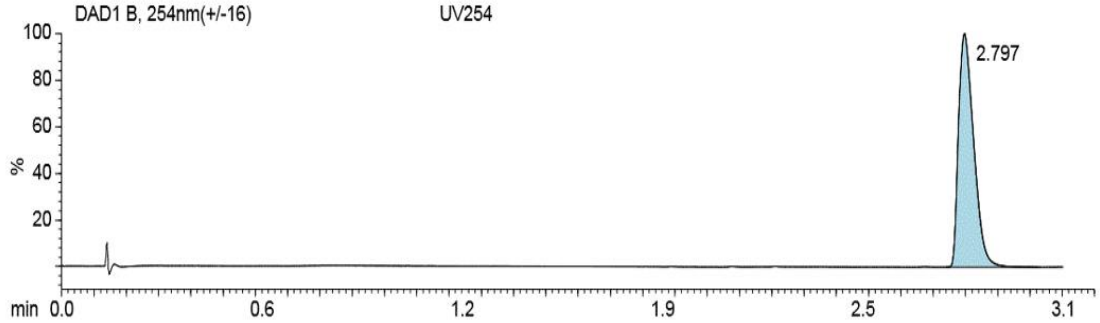

Figure 5. Chromatogram of isolated Compound 3

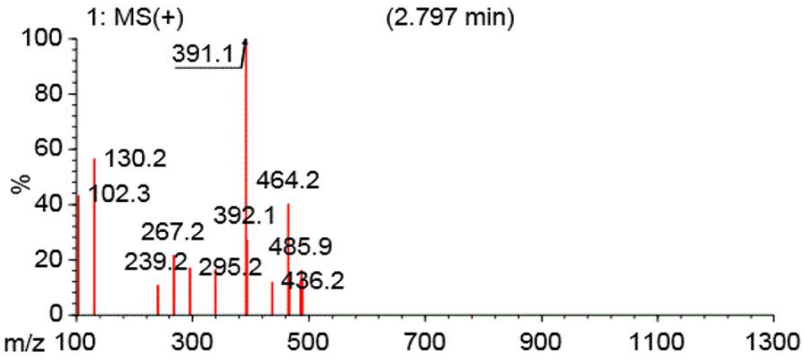

Figure 6. Mass spectral data for Compound 3

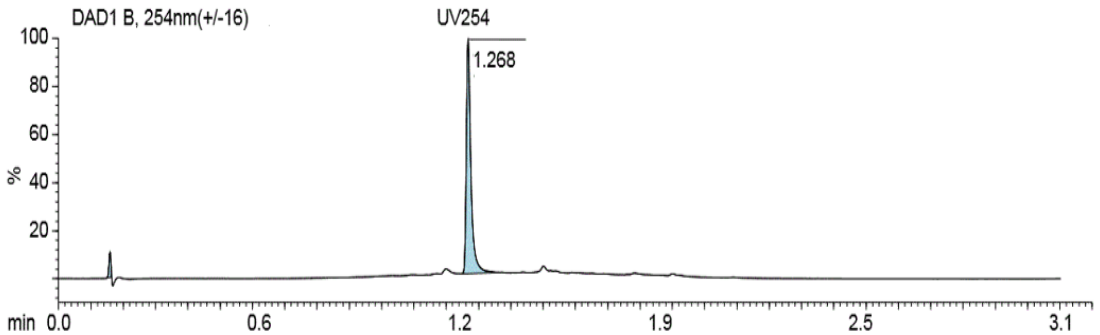

Figure 7. Chromatogram of isolated Compound 4

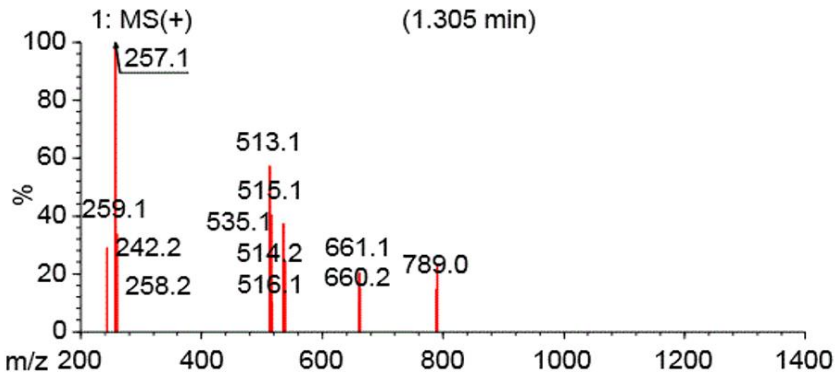

Figure 8. Mass spectral data for Compound 4

Besides showing that the compounds are pure, figures 1,3,5 and 7 provide information about the polarities of the compounds relative to the polarity of the stationary and mobile phases of the liquid chromatography used. In this study, compound 1, 2 and 4 are moderately polar while compound 3 is very non polar. This information becomes handy in predicting the chemistry of the compound and other experiments such as compound preparation for Nuclear Maganetic Rebsonance (NMR) experiments. It would be important to know what solvents need to be used to have the compound fully dissolved. The information also becomes important when considering medicinal chemistry aspects of the isolated compounds, though not known yet of their structures, polarity would be important to predict how good or bad the compound would be if considered for oral administration. Usually, moderate polarity is more preferable for oral drugs unless modifications are to be sought. Although not providing all the information to deduce a complete identification of the compounds, figures 2,4,6 and 8 provide the mass to charge ratios of the compounds. From here, it can be narrowed down to a smaller number of options that could fit the description of the compounds by mass in gram per mole. The masses of the isolated compounds were 127.1, 294.2, 390.1 and 256.1g/mol respectively.

Cytotoxicity Assays of the Isolated Compounds

Isolated compounds showed cytotoxicity against MCF-7 and HepG2 cell lines as shown by IC50 values in figure 9. As can be noticed, doxorubicin retained its highest activity against both cell lines as compared to compounds 1 to 4. The next highest activity was observed in compound 3 against MCF-7 with an IC50 of less than 4 ug/ml. When combined in pairs, a combinatory dose of compounds 2 and 3 was the most active against HepG2 (IC50 = 3.1 ± 0.16 ug/ml) while that of compounds 3 and 4 had the highest activity against MCF-7 with an IC50 of 3.82 ± 0.06 ug/ml.

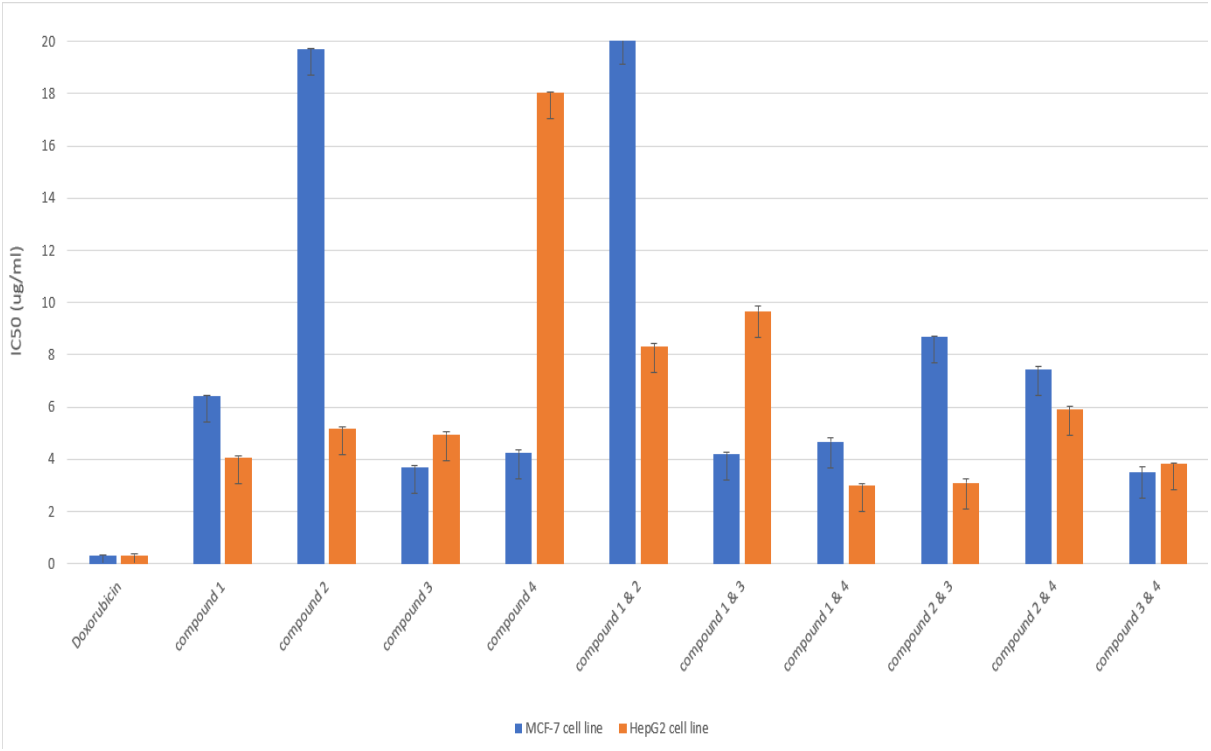

Figure 9. Cytotoxicity of the isolated Compounds on MCF-7 and HepG2 cell lines

To the contrary, when compounds 1 and 2 were administered in combination to both cell lines, the activity reduced as can be observed in Figure 9. The findings in this study showed that combinatory dosing does not always have an additive effect in bioactivity but can rather go either way or remain the same. Figure 10 summarises the net effect of combinatory dosing on both cell lines by colours, where only 40.6% of the tests showed increased activity, 34.4% showed reduced activity and there was no change in activity in 25% of the tests.

	Isolate 1		Isolate 2		Isolate 3		Isolate 4	
	MCF7	HepG2	MCF7	HepG2	MCF7	HepG2	MCF7	HepG2
Isolate 1								
Isolate 2								
Isolate 3								
Isolate 4								

Brown colour denotes reduced activity,
Green colour denotes increased activity,
White colour denotes no change in activity.

Figure 10. Summary of the effect of combinatory dosing on cell lines.

This implies that there is need for logical strategies when considering combinatory therpaies in order to achieve effective results with minimal costs. In addition, some unthoughtful combination of drugs can lead to serious adverse events that could be risky to life(Nakamura & Koo, 2020). The current work agrees with Jawaad and others that even some well designed combinatory therapies do not always gurantee positive clinical outcomes(Fares et al., 2020). From this limited data, there are higher chances of having an additive activity using combinatory dosing of compounds that are known to be active. Therefore, combination therapy in natural products is a viable approach to be explored further in order to curb drug resistance (Derakhshani et al., 2020; Fares et al., 2020), as other individual drugs are being

sought. In natural product drug discovery, the advantage of working with purified compounds is that it is possible for researchers to troubleshoot, replace and monitor the effects of a particular compound which would not be possible if working with a large number of compounds in a sample of an extract, some of which would even be toxic.

Conclusion

Combination therapy does not always lead to a single direction of bioactivity, it can always increase, decrease or maintain bioactivity as it has been shown in this study. There are various factors that lead to increased bioactivity when active compounds have been combined and administered at the same time which amounts to synergism. In synergism, the net effect of all biochemical activities is positive towards reducing a targeted adverse event such as a disease. However, combined drugs or their metabolites may also work in competition which may lead to reduced therapeutic effect and/or an emergence of a different adverse event. It is always important therefore to fully understand the nature of individual compounds being administered in a dose, their potential biological targets and also their mechanisms of action to predict synergism or competitions. Drug discovery in phytochemistry would therefore make more sense if it were viewed beyond the stage of dealing with extracts as an end rather than a step towards getting individual compounds to be considered for combination dosing.

Conflict of interests

Authors declare no conflicting interests

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