**Reviewer’s Comments**

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# CYTOTOXIC ACTIVITIES IN VITRO OF FLOWER EXTRACTS OF THREE SPECIES OF *ALOE* GROWING IN YEMEN:*ALOE RUBROVIOLACEAE, ALOE VERA* AND *ALOE SABAEA*, AGAINST ELEVEN TYPES OF CANCER CELL LINES

**ABSTRACT**

**Background and aims**: Natural products, especially plant extracts, have opened up great opportunities in the field of drug progress due to their chemical variety. The genus *Aloe* has long been used for medicinal uses in countless parts of the world. This study was designed to investigate the phytochemicals and anti-cancer capabilities of *Aloe rubroviolaceae, Aloe vera* and *Aloe sabaea* flowers. **Materials and Methods**: The methanolic extracts of three types of plants traditionally used in Yemen to treat a variety of diseases have been tested in vitro for their potential anticancer activity on different human cancer cell lines. The cytotoxic activity of the methanolic extracts of tested plants was determined using eleven strains of human cancer cells, namely: MCF-7(breast cancer), PC-3 (prostate cancer), HEP-2(human epithelial carcinoma), MNFS-60 (myelogenous leukemia), CACO (intestinalcancer), A-549 (lung adenocarcinoma),HeLa (cervical cancer),RD (rhabdomyosarcoma**) ,** HepG2 (hepatocellular carcinoma), HCT-116 (colon cancer), and CHO-K1(Chinese hamster ovary). A colorimetric sulforhodamine B assay was used to evaluate the in vitro cytotoxic activity of different extracts. Growth inhibition of 50% (IC50) for each extract was calculated from the optical density of treated and untreated cells. Doxorubicin, a broad-spectrum anticancer drug was used as a positive control. **Results:** More interesting cytotoxic activity was observed for *Aloe vera* extract more than *Aloe sabaea and Aloe rubroviolaceae, extract*. **Conclusions:** This study provides a preliminary screening for anti-proliferative activity of various *Aloe* species flowers extracts on different cancer cell lines. Different extracts of *Aloe* speciessignificantly inhibit the growth of various cancer cell lines in a concentration-dependent manner. Further investigations are required to understand the possible mechanism(s) of action of these extract on various cancer cells and isolation of active phyto-chemicals.

# KEY WORDS: *Aloe* species*,* cytotoxic activities, flower extracts, in vitro, Yemen.

**INTRODUCTION**

*Aloe* is a genus that contains more than 550 species of flowering succulent plants. The most widely known species is *Aloe vera*, or "true *Aloe*." It is described this because it is grown as a standard source for various pharmaceutical purposes. These species, as *Aloe rubroviolaceae*, *Aloe vera*, and *Aloe sabaea* are grown or harvested from the wild for similar applications 1-3. The genus is inhabitant to tropical regions, South Africa, Madagascar, Jordan, the Arabian Peninsula including Yemen and is endemic to a variety of islands in the Indian Ocean such as Yemeni Socotra, Reunion, Mauritius, and the Comoros Islands. A few species have also been adapted in other areas such as the India, Mediterranean, South and North America, Australia, and the Hawaiian Islands 4. Most varieties of *Aloe* have a rosette of large, thick, fleshy leaves. The flowers of the *Aloe* are tubular, often yellow, orange, pink, or red, and bush, densely clumped and drooping, at the apex of simple or branching stems devoid of leaves. Many varieties of *Aloe*  appear without stems, with the rose growing directly at ground level; Other varieties may have a branched or un-branched stalk from which the fleshy leaves sprout. They vary in color from gray to light green, and are sometimes striped or speckled. Some species of *Aloe*  native to South Africa are tree-like (*arborescent*) 5. *Aloe* species are often grown as an ornamental plant in both gardens and pots. Many types of *Aloe* are very decorative and are appreciated by collectors of succulents. *Aloe vera* is used internally and externally on humans as a folk or alternative medicine. *Aloe* species is known for its medicinal and cosmetic properties. About 75% of *Aloe* species are used locally for medicinal uses in addition to other herbal plants 6-9. In Yemen, recent researches investigated the effect of herbal plants on viral and bacterial agents and protozoa in which traditional medicine and flavors are used in Yemen where a large number of people rely on herbal plants to treat their diseases 10,11.

It is estimated that in 2018, there were 18.1 million new cases of cancer and 9.6 million deaths worldwide . About 20% of males and 17% of females will develop cancer at some point while 13% of males and 9% of females will expire from it. In 2008, roughly 12.7 million cancers were diagnosed and in 2010 just about 7.98 million people died. Cancers account for about 16% of deaths. The most common as of 2018 are lung cancer (1.76 million deaths), colorectal cancer (860,000), stomach cancer (780,000), liver cancer (780,000) and breast cancer (620,000) 12,13. According to the limited Yemeni Cancer Studies, the most common types of cancer among Yemeni children and adults were leukemia (33.1%), lymphoma (31.5%), central nervous system tumors (7.2%), and bone tumors (5.2%) 14-18. The potential for natural products to be used as a source of anti-cancer agents was accepted in the 1950s by the United States (the National Cancer Institute, NCI) is led by the late Dr. Jonathan Hartwell. NCI has made it a major contributions to the discovery of new naturally occurring anticancer agents through its holding and grant support, including an important program for plant and marine groups. Anti-cancer drugs, such as indole alkaloids. Vincristine, vinblastine, podophyllotoxin derivatives etoposide and teniboside are prominent chemical treatments of plant origin obtained either directly through isolation or derived from lead structures 19. *Aloe* species that originated in the Arabian Peninsula are well recognized for their medicinal use and outside of this species. The traditional uses of *Aloe* species include wound and burn healing and topical treatment of skin diseases 20. Several researchers have also uncovered the role of *Aloe* species in treating eye infections, stomach ailments, constipation and malaria 21,21. The antimicrobial effect of *A. perryi* has also been reported23. Therefore, screening of higher plants for anticancer agents has been pursued on an international level, 24. Yemen is characterized by its vast area, where variations in climate appear due to differences in elevations, which results in a great diversity of its plants. Yemen's botanicals are known for their use in folk and traditional medicine25,26. Thus, this study aims to explore the antiproliferative potential of several *Aloe* species flowers against several human cancer cell lines.

**MATERIALS AND METHOD**

Sample collection and Identification: The flowers of *Aloe Rubroviolaceae and* Aloe Vera were collected fromsaber area in the city of Taiz and Badan mountains (Ibb) respectively, while flowers of Aloe Sabaea were collected from Miatam and Aldlel villages ( Ibb). The taxonomy work and identification of the plants was confirmed by Professor: Abdul Walli Al-Kholidy, Department of Botany, Faculty of Agriculture, Sana'a University, Yemen. The flowers of all plants were collected during the flowering stage in November 2017.

Preparation of Samples: The flowers of the three plants were dried separately in the air and ground to a coarse powder. The powder was then stored in airtight containers at room temperature until use.

**Preparation of ethanol extracts:** The dried flower powder was extracted separately by soaking in sufficient amount of ethanol (99%) with repeated shaking for 1 week and filtered. Each flower filtrate was evaporated and dried separately under reduced pressure at 45 °C using a rotary evaporator (Buchi Rotavapor R-200, Serial No. 05009474, Switzerland) and the process was repeated twice until the extraction was complete.

**Phytochemical screening:** TLC technology was used to identify the components of ethanolic extracts of plant flowers using a TLC plate coated with 60 F254 silica gel, 20 × 20 cm (Merck, Germany). The first developed chromatograms were examined under UV light (VilberLourmat, French) at wavelengths of 254 nm and 365 nm. Then each chromatogram was analyzed for the presence of biologically active components by spraying with appropriate reagents.

**Cytotoxicity assay:** The cytotoxicity of the extracts was tested against eleven types of cancer cell line , Human hepatocellular carcinoma (HepG2), human colon cancer (HCT-116), human breast cancer (MCF-7), human lung adenocarcinoma (A-549), human prostate cancer (PC3), human epithelial carcinoma (HEp-2) and humancervical cancer (HELA), myeloid leukemia (M-NFS- 60), human epithelial colorectal adenocarcinoma (CACO-2), chinese Hamster Ovary (CHO-K1),and rabdomyosarcoma(RD). Cells were obtained from the American Type Culture Collection (ATCC). Tumor cells were propagated in RPMI-1640 medium supplemented with 10% heat inactivated fetal bovine serum, 1% L-glutamine, HEPES buffer and 50 μg/ml gentamicin. All cells were maintained at 37 °C in a humidified 5% CO2 and sub-cultured twice a week.

**Evaluation of cellular cytotoxicity:** The tested cell lines were seeded in a 96-well plate at a cell concentration of 1 × 104 cells per well in 100 μl of growth medium. Fresh medium containing different concentrations of plant extract was added 24 hours after sowing. Serial two-fold dilutions of the test chemical compound were added to conﬂuent cell monolayers dispensed into 96-well, ﬂat-bottomed microtitre plates (Falcon, NJ, USA) using a multichannel pipette. Microtiter plates were incubated at 37 °C in a humidified incubator with 5% CO2 for 48 h. Three wells were used for each test sample concentration. Control cells were incubated without test sample and with or without DMSO. At the end of the incubation period, the production of viable cells was determined by a colorimetric method. Briefly, media was aspirated and crystal violet solution (1%) was added to each well for at least 30 min. The stain was removed and the dishes rinsed with tap water until all excess stains were removed. Glacial acetic acid (30%) was then added to all wells and mixed well, and the absorbance of the plates was then measured after gentle shaking on a microplate reader (TECAN, Inc.), using a test wavelength of 590 nm. The absorbance was proportional to the number of surviving cells remaining in the culture plate. All results were corrected for background absorbance detected in the wells without adding dye. The treated samples were compared with the cellular control in the absence of the tested compounds. All experiments were performed in triplicate. The effect on cell growth was calculated as the difference in the absorbance ratio in the presence and absence of the tested extracts and shown in the dose-response curve. The concentration at which cell growth was inhibited to 50% of the control (IC50) was obtained from the dose-response curve.

STATISTICAL ANALYSIS

The percentage cell viability was calculated using the Microsoft Excel®. Percentage cell viability was calculated as follows:

%Cell viability = (Mean Abs of control) ( Mean Abs of test metabolite) **/** (Mean Abs control) x100

Where: Abs: absorbance at 590 nm.

ETHICAL APPROVAL

Ethical approval was obtained from the Medical Research and Ethics Committee of the College of Medicine and Health Sciences, Sana’a University with reference number (121) on 13/02/2017.

**RESULTS**

Cytotoxicity of ethanolic extracts of flowers from three species of *Aloe* against eleven cancer cell lines (MCF-7, PC-3, HEP-2, MNFS-60, CACO, A-549, HELA, RD, HepG2, HCT-116, and CHO-K1) using the Crystal Violet Staining Assay and evaluated according to American Cancer Institute guidelines. Figures 1-11 and Table 1 show the results and reveal that *Aloe Vera* flower extract showed significant dose-dependent cytotoxic activity against four cell lines (CACO, A-549, RD, HCT-116) with IC50 values ​​of 14.6 ± 0.65, 12 ± 0.50 and 14.7 ± 1.88 and 14 ± 0.46, respectively. However, *Aloe Sabaea* flower extract exerts a very low cytotoxic activity against cell lines, compared to *Aloe Vera* extract. IC50 indicated that the most cytotoxic effect of *Aloe Vera* extract was against the A-549 cell line (IC50 = 43.6 ± 3.07). On the other hand, *Aloe Rubroviolaceae* flower extract showed little cytotoxic activity against three cell lines (CACO, A-549, HCT-116) with IC50 = 30.1 ± 0.95, 26.5 ± 1.05 and 29.7 ± 0.78, respectively. In the present study, the cytotoxic activity was mainly observed in the ethanolic extract of *Aloe Vera* flowers, which showed the highest cytotoxic activity followed by the *Aloe Rubroviolaceae* flower extract, which showed moderate cytotoxic activity, while the *Aloe Sabaea* flower extract showed the lowest cytotoxic activity.

**DISCUSSION**

Since the foundation of human history, innate products have been used for medicinal uses to treat a variety of diseases including cancer 27. Many chemoprophylaxis are molecules derivative from plant resources or their synthetic analogues 28. Vegetable lands were the most a significant foundation and at this time, around 60% of the drugs used to treat cancer have been isolated from natural products, as vincristine and vinblastine from Catharanthus roseus 29, Camptothecin from Camptothica acuminate 30, Taxol and docetaxel from Taxus Previfolia 31. Fruits and vegetables are also known to reduce the risk of cancer in humans32, 33. Some isolated compounds showed anticancer potential with low toxicity compared to conventional drugs, for example. Meisoindigo, isolated from the Chinese plant Indigofera tinctoria and flavopiridol, isolated from the Indian Dysoxylum binectariferum tree34.

Regarding the results of the current study, cytotoxicity evaluation showed that low concentration of *Aloe rubroviolaceae, Aloe vera and Aloe sabaea* extracts significantly inhibited cell proliferation of CACO, A-549, RD and HCT-116 cell lines with a decrease in IC50. The results are in agreement with those of Al-Oqail *et al*. 27,who obtained a dose-dependent response at different concentrations on HEp2, MCF-7, WISH and *Vero* cells. Our results also showed that CACO, A549, RD, and HCT-116 cells were most sensitive to *Aloe Vera* among all studied cell lines and among the extracts, the highest inhibition of methanol extract was found in the A549 cell line with (IC50 = 12 μg/ml), followed by with HCT-116 (IC50 = 14 mcg/mL), RD (IC50 = 14.7 μg /mL), and CACO (IC50 = 14.6 μg /mL).This type of variability between different cell lines was also reported by Heo *et al.*35, who reported the anticancer effects of the plant extract on HEK-293, HCT-116, HeLa, MCF-7, Hep3B, SNU-1066 and SNU-601 cell lines.

In an additional study, the discrepancy cytotoxic response of diverse cancer cell lines (HeLa, HepG2, MCF-7, CACO-2, and L929) was also reported and it was concluded that the plant extract efficiently inhibited cell proliferation depending on the concentration of the extract, also as types of cells 36. The current results are also consistent with previous findings, that plant extracts reduce cell viability in human breast cancer (T47D) cells, due to the sensitivity of cancer cells to lethal flavonoids 37. Furthermore, the growth-inhibitory consequence of components of *Aloe Vera* species in plants has also been confirmed in human uterine cancer (HeLa), melanoma (B16F10), human gastric cancer (MK-1)38 and in other human cancer cell lines39-41. This growth-inhibitory activity may be a consequence of the plant extracts' ability to inhibit DNA synthesis as measured by incorporation of thymidine tritate into cells 42, leading to cell death 43.

**CONCLUSION**

In conclusions, this study presents the phytochemical analysis and preliminary examination of the anti-proliferative activity of extracts of different types of *Aloe* species on different cancer cell lines. We showed that different extracts of *Aloe rubroviolaceae*, *Aloe vera* and *Aloe sabaea* significantly inhibited the growth of different cancer cell lines (CACO, A-549, RD, HTC-116) in a concentration-dependent manner. Among all extracts, *Aloe vera* extract showed the greatest activity and A-549, HCT-116 and RD cells were the most sensitive. In addition, the presence of phytochemicals such as phytosterols, phenols, flavonoids, proteins and glycosides has been confirmed. Further investigations are needed to understand the possible mechanism(s) of action of these extracts on different tumor cells and to isolate the active phytochemicals.

**CONFLICT OF INTEREST**

"No conflict of interest associated with this work”.

**AUTHORS’ CONTRIBUTIONS All authors** contributed equally to the design, implementation, statistical analysis and manuscript drafting. All authors read and approved the final manuscript.

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Figure 1: Percentage inhibition of cell growth of Aloe Vera (AV), Aloe Sabaea (AS), Aloe Rubroviolaceae (AR) extracts and Doxorubicin (DO) against the HCT-116cell line.

Figure 2: Percentage inhibition of cell growth of Aloe Vera (AV), Aloe Sabaea (AS), Aloe Rubroviolaceae (AR) extracts and Doxorubicin (DO) against the HpeG2 cell line.

Figure 3: Percentage inhibition of cell growth of Aloe Vera (AV), Aloe Sabaea (AS), Aloe Rubroviolaceae (AR) extracts and Doxorubicin (DO) against the MCF-7 cell line.

Figure 4: Percentage inhibition of cell growth of Aloe Vera (AV), Aloe Sabaea (AS), Aloe Rubroviolaceae (AR) extracts and Doxorubicin (DO) against the PC3cell line.

Figure5: Percentage inhibition of cell growth of Aloe Vera (AV), Aloe Sabaea (AS), Aloe Rubroviolaceae (AR) extractsand Doxorubicin (DO) against the HEP-2cell line.

Figure 6: Percentage inhibition of cell growth of Aloe Vera (AV), Aloe Sabaea (AS), Aloe Rubroviolaceae (AR) extractsand Doxorubicin (DO) against theMNFS-60 cell line.

Figure7: Percentage inhibition of cell growth of Aloe Vera (AV), Aloe Sabaea (AS), Aloe Rubroviolaceae (AR) extractsand Doxorubicin (DO) against theCACO cell line.

Figure 8: Percentage inhibition of cell growth of Aloe Vera (AV), Aloe Sabaea (AS), Aloe Rubroviolaceae (AR) extractsand Doxorubicin (DO) against theCHO-K1 cell line.

Figure9: Percentage inhibition of cell growth of Aloe Vera (AV), Aloe Sabaea (AS), Aloe Rubroviolaceae (AR) extracts and Doxorubicin (DO) against theA-549 cell line.

Figure10: Percentage inhibition of cell growth of Aloe Vera (AV), Aloe Sabaea (AS), Aloe Rubroviolaceae (AR) extracts and Doxorubicin (DO**)**  against theHELA cell line.

Figure11: Percentage inhibition of cell growth of Aloe Vera (AV), Aloe Sabaea (AS), Aloe Rubroviolaceae (AR) extracts and Doxorubicin (DO) against theRD cell line.

Table 1:In vitro cytotoxic activities of Aloe Vera (AV), Aloe Sabaea (AS), Aloe Rubroviolaceae (AR) extracts against various carcinoma cell lines.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Cell Lines | *Aloe Vera* | *Aloe Sabaea* | *Aloe Rubroviolaceae* | Doxorubicin |
| MCF-7 | 60.3 ±5.17 | > 100 | 89.5 ± 14.9 | 0.35 ± 0.02 |
| PC-3 | 46.1 ± 6.07 | > 100 | 58.3 ±1.57 | 1.68± 0.15 |
| HEP-2 | 62.3 ± 3.19 | >500 | > 100 | 3.58 ± 0.11 |
| MNFS-60 | > 100 | > 100 | > 100 | 1.99 ± 0.12 |
| CACO | 14.6 ± 0.65 | 92 ± 5.81 | 30.1 ±0.95 | 1.71 ± 0.03 |
| A-549 | 12 ± 0.50 | 43,6 ± 3.07 | 26.5 ±1.05 | 0.95 ± 0.16 |
| HELA | > 100 | >500 | > 100 | 3.56 ± 0.12 |
| RD | 14.7 ± 1.88 | > 100 | 61.8 ± 4.44 | 6.07 ± 0.22 |
| HepG2 | 52.4 ± 4.79 | > 100 | 55.7 ±3.09 | 0.36 ± 0.02 |
| HCT-116 | 14 ±0.46 | 51.3 ± 4.08 | 29.7 ±0.78 | 0.49 ± 0.04 |
| CHO-K1 | > 100 | > 100 | > 100 | 0.84 ± 0.06 |

Cytotoxic activity is expressed as IC50 (μg/mL)**±**SD (n **=** 3), which is the concentration of extract at which 50% of cell growth was inhibited relative to cellsincubated in the presence of**<**0.1% DMSO vehicle control. All cell lines were treated with doxorubicin as a positive control. MCF-7(breast cancer), PC-3 (prostate cancer), HEP-2(human epithelial carcinoma), MNFS-60 (myelogenous leukemia)**,** CACO (intestinalcancer), A-549 (lung adenocarcinoma),HeLa (cervical cancer),RD (rhabdomyosarcoma**) ,**HepG2 (hepatocellular carcinoma), HCT-116 (colon cancer), and CHO-K1(Chinese hamster ovary**) .**

**Author reply to some comments**

Comment 13K:

The phytochemical screening is left aside without corroborating with the in vitro cell line results. Need clarity in results and discussion presentation.

Author’s answer: These results will be presented and discussed in an additional article shortly.