Achillea fragrantissima (Forssk.) Sch.Bip. methanolic extract exerts potent antimicrobial activity and causes cancer cell death via induction of caspase-dependent apoptosis and S-phase arrest

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\textbf{ABSTRACT}
This study was done to evaluate the anticancer potential of Achillea fragrantissima (Forssk.) Sch.Bip. leaves methanolic extract in detail for the first time, in addition to investigating its antimicrobial activity. The antimicrobial assay revealed that the extract exerted high activity against \textit{P. vulgaris} (MIC = 156.25 \textmu g/ml) and \textit{C. albicans} (MIC = 625 \textmu g/ml), while moderate activity was observed against other microbes. The extract was also screened against HepG2, A549, HCT116 and MCF7 cancer cells and was found to be active across all cells with highest selectivity and cytotoxic activity being observed for A549 cells (IC\textsubscript{50} = 1.21 \textmu g/ml). Further mechanistic studies on A549 cells showed that the extract resulted in S-phase arrest and induced apoptosis via activation of caspase-3, p53 and Bax, in addition to downregulation of Bcl-2. HR-LCMS analysis indicated the presence of 3-hydroxycoumarin, quercetin 3,3\textsuperscript{0}-dimethyl ether and skullcapflavone II which might be responsible for the extract’s bioactivity.

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1. Introduction

Achillea fragrantissima (Forssk.) Sch.Bip. is a plant that is commonly found in several Arab countries and has been traditionally used to treat gastrointestinal disorders, eye infections, smallpox and diabetes (Awad et al. 2017). Further pharmacological work have shown that the plant exerted antimicrobial activity in addition to cytotoxic activity against several cancer cell lines, such as leukaemia and liver cancer cells (Alenad et al. 2013; Hammad et al. 2014; Awad et al. 2017).

Although several antimicrobial and anticancer studies were performed on Achillea fragrantissima, they are still considered inadequate. For instance, the antimicrobial studies were mostly conducted on the plant’s essential oil (Alsohaili 2018), while very few studies involved the plant’s organic or aqueous extracts (Hammad et al. 2014). Moreover, even fewer studies have investigated the antimicrobial activity of the plant’s methanolic extract in a comprehensive manner (Elsharkawy et al. 2021). Similarly, the studies performed on Achillea fragrantissima’s cytotoxic activity were very preliminary and often involved screening the plant’s extracts against a panel of cancer cell lines without further studies involving the mechanism of action (Alenad et al. 2013; Hammad et al. 2014).

Therefore, the main aim of the present study is to investigate the antimicrobial and anticancer potential of the methanolic extract of Achillea fragrantissima leaves from Saudi Arabia’s region of Hail in a detailed and comprehensive manner for the first time, in addition to elucidating its anticancer mechanism of action.

2. Results and discussion

2.1. Antimicrobial activity of Achillea fragrantissima methanolic extract

Achillea fragrantissima leaves methanolic extract was initially screened against certain microbes using a qualitative agar well diffusion assay (supplementary material Table S1). Results showed that the methanolic extract exerted moderate antimicrobial activity against all investigated microbes except for P. vulgaris and C. albicans whereby significantly high antimicrobial activity was exerted with inhibition zones of 24.1 mm and 14.7 mm, respectively. It is interesting to note that the inhibition zone for P. vulgaris was similar to that of gentamycin, indicating the antimicrobial potential of the extract against this specific type of bacteria. Moreover, the fact that the extract was moderately active against the other examined microbes further proves its antimicrobial potential in general.

Further quantitative analysis of the extract’s antimicrobial activity was performed by investigating the extract’s minimum inhibitory concentration using the broth microdilution assay (Supplementary material Table S2). Results of the assay corroborated those of the well diffusion assay, whereby the extract was found to be most active against P. vulgaris followed by C. albicans with MIC values of 156.25 µg/ml and 625 µg/ml, respectively. Moreover, the MIC value of the extract against C. albicans was found to be comparable to that of the positive control ketoconazole. Therefore, it can be deduced that the extract possesses specifically high activity against P. vulgaris followed by C. albicans.
2.2. *In vitro anticancer evaluation of Achillea fragrantissima* methanolic extract

The anticancer potential of *Achillea fragrantissima* has not been investigated comprehensively so far, therefore, we decided to examine the cytotoxic activity of *Achillea fragrantissima* leaves methanolic extract and elucidate its mode of action for the first time.

The methanolic extract of *Achillea fragrantissima* has been screened against certain cancer cell lines (supplementary material Table S3). Results have shown that the extract exerted high cytotoxic activity across all cell lines with IC\(_{50}\) values close to that of vinblastine sulfate. The highest cytotoxic activity was exerted against A549 cells with an IC\(_{50}\) value of 1.21 \(\mu\)g/ml, and it is interesting to note that in this case the extract possessed higher activity than that of vinblastine sulfate. Moreover, the extract showed more selectivity towards all cancer cell lines relative to normal, healthy MRC5 cells, with highest selectivity being demonstrated against A549 cells (supplementary material Table S4). These interesting results with A549 cells specifically, encouraged us to further investigate the mechanism of action of the extract in A549 cancer cells.

We wanted to further characterise the extract’s bioactivity against A549 cells via investigating its effect on cell-cycle progression. A549 cells treated with the extract showed an increase in the fraction of cells in the S-phase where DNA replication occurs (supplementary material Figure S1). Moreover, a significant increase in the fraction of cells in the pre-G1 phase was also observed after treatment with the extract which indicates that the extract induces apoptosis in A549 cells. Therefore, the extract induced S-phase arrest and apoptosis in A549 cells.

Cell-cycle analysis indicated that the extract induced apoptosis in A549 cells. Therefore, to further investigate the induction of apoptosis, an Annexin V/propidium iodide (PI) apoptosis assay was conducted. Results showed that the extract induced early and late apoptosis (supplementary material Figure S2). Moreover, there was also an increase in the number of necrotic cells after treatment. Therefore, it can be deduced that the extract resulted in cancer cell death mostly via the induction of apoptosis while a less percentage of cells were found to have undergone necrosis.

The induction of apoptosis by the methanolic extract may be further confirmed via investigating the expression levels of apoptosis-related proteins, such as caspase-3 and p53. Western blot analysis (supplementary material Figure S3) showed an increase in the protein expression levels of cleaved caspase-3 after treating A549 cells with the methanolic extract, which indicates the induction of apoptosis in the cancer cells upon treatment and corroborates the data obtained from the Annexin V/PI assay. Moreover, p53 expression levels were found to be enhanced following treatment with the methanolic extract which might indicate that the induced apoptosis is probably mediated via p53.

Bax is a pro-apoptotic protein that is a primary target of p53 and is responsible for caspase activation, however, the pro-apoptotic effects of Bax are suppressed by the anti-apoptotic protein Bcl-2. Western blot analysis revealed that the methanolic extract increased the protein expression level of Bax but reduced the expression level of Bcl-2 in A549 cells (supplementary material Figure S3), which further indicates the pro-apoptotic effects of the extract. Therefore, the extract was found to cause apoptosis via activating caspase-3, p53 and Bax in A549 cells.
2.3. HR-LCMS analysis

The methanolic extract was analysed via HR-LCMS in order to identify its phytochemical constituents and thus provide a plausible explanation to the extract’s observed high bioactivity (supplementary material Table S5). The analysis showed the presence of 3-hydroxyxocoumarin in addition to quercetin 3,3′-dimethyl ether, and these compounds might be the reason behind the observed biological activity of the methanolic extract. 3-hydroxyxocoumarin identified in the methanolic extract might be responsible for the observed antimicrobial and anticancer activity of the extract, as it was previously found to disrupt cell-to-cell signalling of certain bacterial cells (D’Almeida et al. 2017), while other studies have demonstrated its ability to protect against carcinogenesis via induction of anticarcinogenic enzymes (Dinkova-Kostova 2002). On the other hand, quercetin 3,3′-dimethyl ether is thought to have been responsible for the observed anticancer activity of the extract, as it was previously reported that it inhibited the growth of a panel of six cancer cell lines (Pettit et al. 2005). Moreover, skullcapflavone II has also been identified in the extract, and this compound has been previously found to possess antimicrobial/anticancer activity (Solnier et al. 2020; Vetrivel et al. 2021), which suggests that it might have played a role in the observed bioactivity of the extract. It is crucial to note that the compounds identified by HR-LCMS analysis are yet to be isolated as few such studies have been performed on Achillea fragrantissima (Ezzat and Salama 2014). However, compounds such as quercetin 3,3′-dimethyl ether (Valant-Vetschera and Wollenweber 1999) and santin (Abd-Alla et al. 2016; Venditti et al. 2016) have previously been isolated from other species of Achillea, while tetraneurin A (Ramesh et al. 2003), 3-O-Acetylpadmatin (Máñez et al. 1999) and neopellitorine A (Ekiert et al. 2021) were previously isolated from other plants that share the same family, Compositae, as Achillea fragrantissima.

3. Experimental

See supplementary material.

4. Conclusions

In summary, the current study involved assessing the anticancer activity of Achillea fragrantissima methanolic extract in greater detail for the first time, in addition to assessing the extract’s antimicrobial activity. The extract was found to possess moderate antimicrobial activity in general, with high activity specifically observed against P. vulgaris and C. albicans. Moreover, the extract exerted its highest cytotoxic activity against A549 cells and was found to possess high selectivity to these cells relative to normal cells. Further studies showed that the extract caused caspase-dependent, p53-mediated apoptosis in A549 cells and resulted in S-phase cell cycle arrest. Taken together, this study demonstrated the antimicrobial and anticancer potential of Achillea fragrantissima methanolic extract and provided a better understanding about the extract’s anticancer mode of action. It is expected that this work would guide future isolation and in vivo studies so that the extract or one of its chemical constituents would be finally developed as an antimicrobial/anticancer agent. Moreover, there
is also the possibility of examining the antiviral potential of *Achillea fragrantissima* methanolic extract in the future as such studies have not been performed comprehensively so far.

**Disclosure statement**

The authors declare no conflict of interest.

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