As current methods for antibiotic drug discovery are being outpaced by the rise of antimicrobial resistance, new methods and innovative technologies are crucial to replenish our dwindling arsenal of antimicrobial therapeutics. While natural products are a well-studied source of biologically active small molecules, peptidyl factors contributing to their medicinal properties remain largely unexplored. To this end, we have developed the PepSAVI-MS (Statistically-guided bioactive peptides prioritized via mass spectrometry) pipeline to identify bioactive peptide targets from complex biological samples. MS/MS techniques such as CID, ETD, UVPD are implemented for de novo characterization. To validate this pipeline, we have demonstrated successful detection and identification of a known antimicrobial peptide, cycloviolacin O2 (cyO2), from the botanical species *Viola odorata*. Additionally, we have widened the known antimicrobial spectrum for *V. odorata* cyclotides, including antibacterial activity of cyO2 against *A. baumannii* and novel anticancer activities for cycloviolacins by their cytotoxicity against ovarian, breast and prostate cancer cell lines. The developed platform is highly versatile as it is adaptable to any natural product source of peptides and can test against diverse physiological targets, including bacteria, fungi, viruses, protozoans, and cancer cells for which there is a developed bioassay. As such, we demonstrate extension of this pipeline to fungal and bacterially-sourced AMPs through the identification of the killer toxin KP4 from *Ustilago maydis* and the bacteriocin bac-21 from *Enterococcus faecalis* harboring pPD1. Bac-21 is identical in nucleotide sequence to another enterococcal bacteriocin, AS-48, but herein we have experimentally validated the protein sequence of bac-21 for the first time. Additionally, we begin to probe the vast array of botanical natural product sources to prioritize highly active species for downstream analysis.