The advent of mass spectrometry (MS), which is renowned for its sensitivity and selectivity, analytical methods based on, combined liquid chromatography and tandem mass spectrometry (LC-MS/MS) can rapidly establish and determine low levels of drugs and their metabolites in biological fluids. LC-MS/MS analysis offers high sensitivity and applicability to almost every type of compound. Picroliv preparation has been developed by Central Drug Research Institute, Lucknow, from the roots and rhizomes of Picrorhiza kurroa Royle (Scrophulariaceae). It forms a major ingredient of many Ayurvedic preparations prescribed in the treatment of several ailments of liver and spleen, fever and asthma has shown excellent hepatoprotective activity and immunomodulatory action in number of laboratory studies. Picroliv preparation is standardized based on picroside-I and kutkoside. But, the multiple constituents may work ‘synergistically’ and could hardly be separated into active parts. Thus, it deemed necessary to determine most of the phytochemical constituents of picroliv preparation in order to ensure the reliability and repeatability of pharmacological and clinical efficacy. Several chromatographic techniques can be applied for standardization of herbal preparation. We used HPLC-UV and LC-MS analysis for the standardization of picroliv preparation by pattern profiling of various constituents along with a precise and accurate method to estimate relative concentration of major components in the preparation. In total, 27 components could be detected, out of which fourteen could be quantified in terms of their relative concentration. Seven components, including picroside-I and kutkoside, were structurally correlated and confirmed. The method was successfully applied to detect and determine relative concentrations of various components in two different batches of picroliv.