

Separation and identification of berberine in plant extracts using LC-MS for antibacterial activity against E. coli determination and artificial intelligence prediction for other activities

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Abstract

A fast LC-MS method has been developed for the identification of berberine in plant extracts. The retention times for the standard berberine and berberine extracted from *Berberis vulgaris*, *Berberis aquifolium*, and *Hydrastis canadensis* were 1.80, 1.82, 1.79 and 1.79, respectively, using mobile phase of a mixture of ammonium acetate buffer and acetonitrile with gradient mode. The column used Waters Acquity BEH C₁₈ (50 x 2.1 mm, 1.7 µm). The purity of the standard berberine was recorded as 98.86% with desired mass of 336 while these values were 61.82, 69.02 and 49.98% for berberine extracted from *Berberis vulgaris*, *Berberis aquifolium* and *Hydrastis canadensis*. In addition, an artificial intelligence technique was also applied to predict the possible activity of berberine against 27 known diseases. The results indicated berberine as the most active against Dengue larvicide, E. coli, Alzheimer and PTR L Major with 1.0 as the maximum probability. The outcomes reported herein are very important to determine the purity of the plants that extracted berberine in the future. The antibacterial activity of berberine was carried by MTT assay. The IC₅₀ of berberine was calculated 136.3 µM against *E. coli*, while IC₅₀ of a standard kanamycin taken as positive drug control calculated 10.87 µM. Also, the applied artificial intelligence may be extended to predict the biological activity of berberine or more diseases.

1. Introduction

Berberine (Fig. 1) is a very important alkaloid used to treat various diseases [1–13]. The most important source of this alkaloid is various plants; particularly *Berberis vulgaris*, *Berberis aquifolium*, and *Hydrastis canadensis*. The important parts of these plants are roots, rhizomes, and stem bark. It has been utilized for millennia in conventional treatment, particularly in certain Asian traditional medical systems including Ayurveda and Traditional Chinese treatment. Due to its possible biological significance and health advantages, berberine has attracted a lot of attention in contemporary scientific research. Antioxidant, antibacterial, anti-inflammatory, cardiovascular health, blood sugar regulation, neuroprotective benefits, and cancer prevention are just a few of its significant biological properties. Although berberine shows promise for a number of health advantages, a more thorough clinical study is required to determine its efficacy and safety for a variety of medical disorders, which necessitates berberine to be in pure form. The purity is an integral part of the efficacy of any molecule; including berberine.

There are some papers on the separation and identification of berberine by thin-layer chromatography [14, 15] and high-performance liquid chromatography [16]. It has been observed from these papers that the detection limits are not good. Moreover, a critical evaluation of these papers was carried out and observed that these methods used a large time and costly chemicals. This observation leads to compel researchers to explore LC-MS methods. By searching through literature, it was observed that only a few papers are available. Hayati et al. [17] recorded the LC-MS method of berberine in *Acalypha Indica* Linn plant extract. The varied flow rates were used in two mobile phases *i.e.* MeOH: 0.1% TFA (50:50) and ACN:0.1% TFA (60:40 v/v)) on C₁₈ column. Liang et al. [18] reported UPLC-MS/MS using Acquity UPLC® BEH C₁₈ column and detection by a triple quadrupole mass spectrometer with electrospray ionization.

The sample preparation was done within 15 minutes with 3.9-minute LC-MS run. These are the only two available methods and after evaluation, it was analyzed that the further need for LC-MS; especially in plant extracts.

In today's era, artificial intelligence plays a pivotal role in diverse fields, with a notable impact on medicinal chemistry. The use of MolPredictX, an online AI tool, stands out as a valuable resource for forecasting the biological activity of herbs. This not only showcases the growing importance of AI in scientific exploration but also opens avenues for more efficient and informed approaches in medicinal research [19]. Therefore, adopting a forward-thinking strategy, AI powered tool, specifically MolPredictX, has been employed to anticipate the biological activities of berberine. This groundbreaking methodology, encompassing a comprehensive study across 27 diseases, utilizes MolPredictX's sophisticated algorithms to discern probable active or inactive outcomes associated with berberine. Additionally, this approach goes a step further by identifying any known targets with the highest probability, providing an avenue for further investigation and potential repurposing of berberine in the context of medicinal chemistry. This not only enhances our understanding of its activities against specific diseases but also opens doors to targeted and purposeful exploration of its therapeutic potential. Keeping in view, the great demand to develop a more advanced method for the separation and identification of such important alkaloids present in various plants and the application of artificial intelligence, the present paper describes LC-MS separation of berberine in plant extract and the prediction of its activities against several diseases.

2. Materials And Methods

2.1 Experimental

2.1.1 Chemicals and reagents

Methanol, ethanol, *n*-propanol, and acetonitrile of HPLC grade were purchased from Merck, India. Formic acid was procured from Qualigens, India. Standard berberine chloride, DMSO were purchased from Sigma-Aldrich, UA. *E. coli* clinically isolate. MTT, Mueller Hinton and Kanamycin were procured from Hi-Media.

2.1.2 Instrumentation

Shimadzu LC-MS 2020 (LabSolution) with MS detector, single quadrupole, and mass range of 10-2000 *m/z* was used. The MS interface ESI (Electro Spray Ionization) and ESI + APCI (DUIS Mode) were applied. PDA detector having D2 (deuterium) and W(tungsten) was also utilized. Autosampler with a 15000-psi pressure unit and temperature-controlled column oven maximum up to 105°C was adopted. The column used Waters Acquity BEH C₁₈ (50 x 2.1 mm, 1.7 µm). Incubator shaker of Brunswick and 96 plate from Torsion. Plate reader from Spectramax.

2.2 Methodology

2.2.1 Extraction of berberine from plants

The extraction of berberine from *Berberis Vulgaris* (bark of the root), *Berberis aquifolium* (root), and *Hydrastis canadensis* (rhizome) plants was carried out as reported by us earlier [20].

2.2.2 Sample Preparation

The standard berberine 1.0 mg.mL^{-1} was prepared in methanol. Whereas the plant-extracted samples were prepared in a mixture of ethanol and water (70:30, v/v). These samples were filtered through a $0.45 \mu\text{m}$ membrane. The subsequent dilution as per the requirement was carried out in methanol and used in LC-MS studies.

2.2.3 Analytical separations procedure

LC-MS experiments were carried out in a gradient mode using ammonium acetate buffer (5.0 mM) and acetonitrile at different flow rates. The experimental conditions are given following Table 1.

Table 1
Gradient mobile phase of LC-MS.

Time	Buffer (%)	Acetonitrile (%)	Flow rates (mL/min)
0.00	95	5	0.6
0.25	95	5	0.6
1.50	65	35	0.6
2.50	5	95	0.8
3.20	5	95	0.8
3.50	95	5	0.6
4.00	95	5	0.6

2.2.4 Bacterial Strain and Growth conditions

Clinically isolated *E. coli* (*Escherichia coli*) patient sample grown in nutrient broth media (Mueller Hinton) at 37°C for 24 hours in incubator shaker.

2.2.5 Biological activity prediction using ML based online tool

In the quest to unravel the biological activities of natural products, MolPredictX, an online tool harnessing machine learning, played a pivotal role. A meticulous exploration was undertaken to unveil the potential activities of berberine against 27 diseases using advanced machine learning techniques. The Simplified Molecular Input Line Entry System (SMILES) notation served as the key to MolPredictX, offering a string-based representation of berberine's molecular structure [21, 22]. To enable MolPredictX's analysis, the

validated SMILES notation of berberine was submitted, confirming its adherence to the correct chemical structure [23]. MolPredictX, driven by machine learning algorithms, meticulously scrutinized the molecular features of berberine. The predictions encompassed a spectrum of biological activities, spanning anti-inflammatory, anticancer, antimicrobial, antioxidant, and other pertinent properties. This sophisticated approach not only expands our understanding of berberine's potential applications but also underscores the importance of accurate molecular representation in leveraging machine learning for predictive analyses.

3. Results and discussions

3.1 Chromatographic separation

The extracted samples were run on LC-MS (LabSolution software) system with the gradient mode in 4.0 minutes of run time. Similarly, a standard sample was run to compare the active marker berberine. A mixture of ammonium acetate buffer and acetonitrile in gradient mode was used as given in the experimental section. The standard peak (marker) was observed at 1.80 retention time. An impurity also appeared at 1.67 retention time. The extracted berberine samples are also run under the identical conditions of LC-MS and the chromatograms of the separated berberine are shown in Fig. 2. The retention time of the standard berberine was 1.80 minutes while the retention times of berberine extracted from *Berberis vulgaris*, *Berberis aquifolium* and *Hydrastis canadensis* were 1.82, 1.79 and 1.79 minutes, respectively. The capacity factor of the standard berberine and berberine extracted from *Berberis vulgaris*, *Berberis aquifolium*, and *Hydrastis canadensis* were 0.075, 0.083, 0.070, and 0.073, respectively. Similarly, the resolution factor calculated for the standard berberine, *Berberis vulgaris*, *Berberis aquifolium*, and *Hydrastis canadensis* were 1.96, 2.49, 1.76, and 1.75, separately and, respectively (Table 2). The number of theoretical plates for the standard berberine, *Berberis vulgaris*, *Berberis aquifolium*, and *Hydrastis canadensis* was 6990, 11383, 4882 and 5584. The tailing factor calculated for the standard berberine, *Berberis vulgaris*, *Berberis aquifolium*, and *Hydrastis canadensis* were 1.49, 1.67, 1.33 and 1.47 separately and, respectively (Table 3). The values of the theoretical plates ranged from 4882 to 11383; indicating good column performance. These values indicate that the reported method of LC-MS is quite good for the separation and identification of berberine in plant samples.

Table 2
LC-MS separation data of standard and extracted berberine.

Compounds	tr (min)	k	Rs
Standard berberine	1.80	0.075	1.96
<i>Berberis vulgaris</i>	1.82	0.083	2.49
<i>Berberis aquifolium</i>	1.79	0.070	1.76
<i>Hydrastis canadensis</i>	1.79	0.073	1.75

Table 3
Theoretical plate and tailing factors of separated berberine in LC-MS.

Compounds	Number of theoretical plates	Tailing factors
Standard berberine	6990	1.49
<i>Berberis vulgaris</i>	11383	1.67
<i>Berberis aquifolium</i>	4882	1.33
<i>Hydrastis canadensis</i>	5584	1.47

3.2 Mass identification

The standard peak (marker) observed at 1.80 retention time showed a desired mass of 336 with a purity of 98.86%. An impurity corresponding to $[M+1]$ peak at 1.67 retention time showed a mass of 338 with the main peak of berberine (336 mas); with 1.14% of purity. The marker showed unknown impurity mass of 671 at the same retention time (Fig. 3). Similarly, herb-extracted samples were run and observed a peak at a similar fashion with a desired mass of 336 followed by $[M+1]$ peak impurity having a mass of 338. The observed purities of berberine extracted from *Berberis vulgaris*, *Berberis aquifolium* and *Hydrastis canadensis* were found to be 61.82, 69.02 and 49.98, respectively. An impurity corresponding to $M+1$ peak (mass 338) was also observed in the extracted samples with 0.67, 0.99 and 1.49% in *Berberis vulgaris*, *Berberis aquifolium*, *Hydrastis canadensis* separately, and respectively.

3.3 Validation of the Methods

The currently developed LC-MS method was validated with respect to various chromatographic phases like linearity, the limit of detection (LOD), the limit of quantitation (LOQ), precision, accuracy, selectivity, robustness and ruggedness.

3.3.1 Linearity

The linearity of calibration curves (peak area vs. concentration) for the berberine standard was calculated over the concentration ranges of 0.10–0.80 $\mu\text{mg.mL}^{-1}$. The calibration graph plotted was linear over these concentration ranges ($n = 5$). The linear regression analysis was determined on the resultant curves. The correlation coefficient (R^2) ($n = 5$) was found to be 0.998 for the standard berberine. The findings of RSD for the standard berberine and berberine extracted from *Berberis vulgaris*, *Berberis aquifolium* and *Hydrastis canadensis* were 1.56 1.88, 1.92, and 1.96.

3.3.2 LOD and LOQ

The outcomes for LOD and LOQ for berberine extracted from *Berberis vulgaris*, *Berberis aquifolium* and *Hydrastis canadensis* were 1.0 and 1.5 $\mu\text{g.mL}^{-1}$, respectively. The RSDs values were 1.56 1.88, 1.92, and 1.96.% for berberine and berberine extracted from *Berberis vulgaris*, *Berberis aquifolium* and *Hydrastis canadensis*.

3.3.3 Specificity

The method is a fine specific which is clear from Fig. 2. The retention times of berberine and berberine extracted from *Berberis vulgaris*, *Berberis aquifolium* and *Hydrastis canadensis* were nearly similar. The calculated results proved a good specificity of the reported LC-MS method.

3.3.4 Precision

The precision value was tested by applying three concentrations of berberine and berberine extracted from *Berberis vulgaris*, *Berberis aquifolium* and *Hydrastis canadensis* were 0.01, 0.05 and 0.10 $\mu\text{g.mL}^{-1}$. Further, five replicate runs were performed for the aforesaid concentrations. RSDs values were 2.00 and 2.70%; showing LC-MS method precise.

3.3.5 Accuracy

Different samples of berberine and berberine extracted from *Berberis vulgaris*, *Berberis aquifolium* and *Hydrastis canadensis* were optimized to check the authenticity for accuracy method as given in the experimental section. The accuracy was done by running five replicates ($n = 5$). The percent error was calculated and found 1.5 to 2.30% in all the cases. The above findings indicated a good accuracy of the developed method.

3.3.6 Robustness and Ruggedness

Small changes were performed such as in mobile phase compositions, flow rates, amounts loaded and detection wavelengths. It was observed that there was no prominent variation in HPLC results, confirming the developed method as robust. The ruggedness was also performed during the development of the LC-MS method.

3.3.6 Intra- and inter-day assays

The RSD values for intra- and inter-day assays were 2.00 and 2.51; showing the robustness of the method. In addition, the results achieved with different operators were unaffected, which also indicated the ruggedness of the method.

4. Antibacterial activity Assay

The antibacterial activity of berberine represented as inhibitory concentration, by MTT Assay. Concentrations of berberine used from 1000 to 100 μM . Before adding berberine to culture *E. coli* cells were seeded in 96 well plate (Fig. 4) at a concentration of 10^5 cells/100 μL /well in triplicate manner. Kanamycin used as a control and treated *E. coli* cells with same concentrations, for negative control broth media was used. After 12 hours of incubation MTT substrate was added finally to check activity at a concentration of 0.2 mg/ml and incubated for 1-hour, resultant formazan was further solubilized using organic solvent DMSO at 570 nm measured using a multiwell scanning spectrophotometer.

Since berberine is showing antibacterial activity against *E. coli* as per artificial intelligence probable activity mentioned in Fig. 4. Based on these prediction findings we have carried out the experimental work. The IC_{50} of active Berberine compound ranging from 100 μM to 1000 μM was based on pilot studies conducted prior to final studies. IC_{50} of Berberine was calculated 136.3 μM (Fig. 5) against *E. coli*, while IC_{50} of a well-known antibacterial agent kanamycin taken as positive drug control calculated 10.87 μM (Fig. 5). All the data analyzed by using GraphPad Prism 8.0.2. software.

5. Prediction of biological activities of berberine

Also, the applied artificial intelligence may be extended to predict the biological activity of berberine or more diseases. MolPredictX offers valuable insights into the biological activities of natural products. The strengths of MolPredictX lie in its ability to rapidly analyze large datasets and provide insights for further investigation. MolPredictX was also used to determine the activity and inactivity of berberine against various diseases. Validation of the predictions was performed by comparing them with available experimental data and other validated methods to assess the performance of MolPredictX. The activity was predicted in terms of probability, probability active, and probability inactive (Fig. 6). It is clear from this Figure that berberine is most active against Pentamidine Resistant *Leishmania major* (PTRL), Alzheimer NADPH, *E. coli* and Dengue larvicide with maximum probability of 1.0. Besides, berberine is also effective against other diseases as shown in Fig. 6. The activity predictions in the experimental findings complemented the literature available [24–26], enhancing our understanding of the bioactive compounds present in plant herbs. The predictions were analyzed in conjunction with this research objectives and compared with available experimental and literature data [27–29], confirming the reliability of the results presented herein.

6. Conclusion

The developed method for the separation and identification of berberine in *Berberis vulgaris*, *Berberis aquifolium*, and *Hydrastis canadensis* plants is fast, accurate, selective, reproducible, and economical with respect to other available methods. The separation and identification of berberine in the plant extracts was achieved within 2 minutes time - a quick method. The purity of the standard berberine was recorded as 98.86% with the desired mass of 336 while these values were 61.82, 69.02 and 49.98% for berberine extracted from *Berberis vulgaris*, *Berberis aquifolium* and *Hydrastis canadensis*. The use of artificial intelligence is another asset of this work. The maximum probability (1.0) of berberine activity was found to be for Dengue larvicide, *E. coli*, Alzheimer and PTR L Major. The reported LC-MS method is useful to determine the purity of berberine in the plants' extracted samples. IC_{50} value of berberine was 136.3 μM against *E. coli*, while standard kanamycin was 10.87 μM . Besides, the application of artificial intelligence may be extended to predict berberine activity against other diseases.

Declarations

Author Contribution

SDA: Methodology, software; IA: Conceptualization, Writing original draft; MAB: Methodology, software; VK: Visualisation, software; PD: Visualisation, software; MB: Visualisation, software; ML: Conceptualization, Writing original draft; ZAA: Conceptualization, Writing original draft.

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Figures

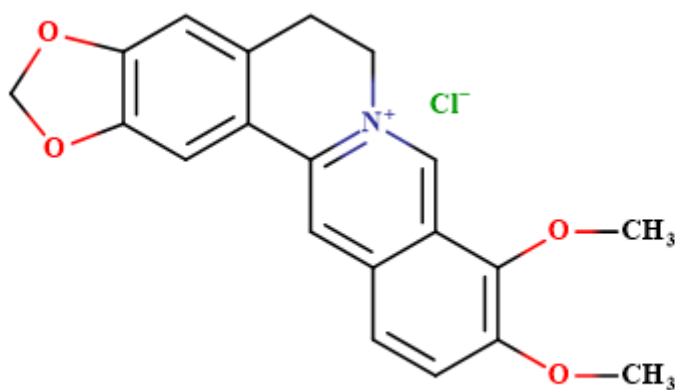


Figure 1

Chemical structure of berberine chloride.

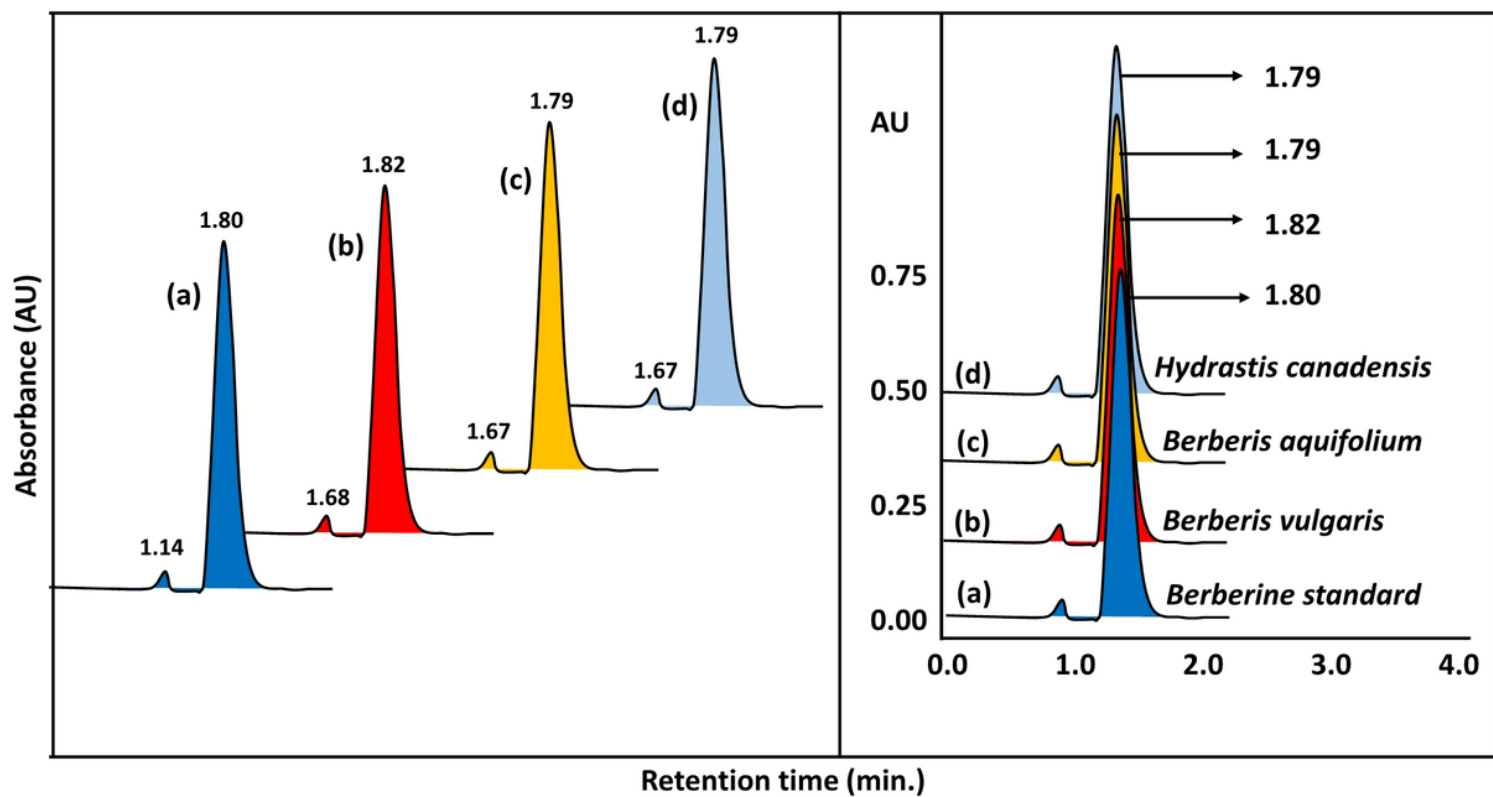
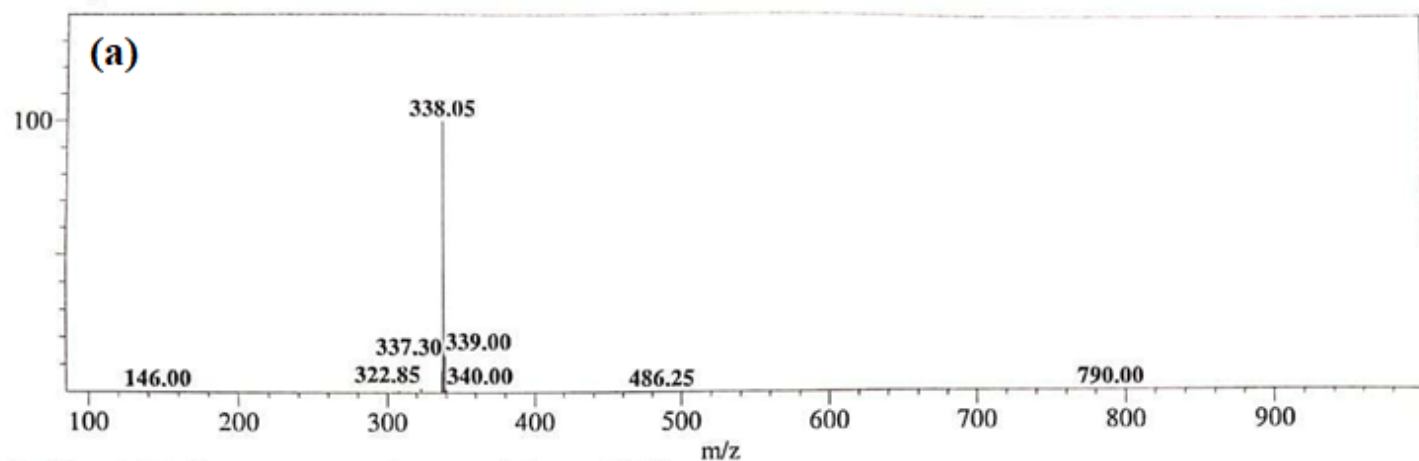


Figure 2

Chromatograms of berberine, (a): standard, (b): *Berberis vulgaris*, (c): *Berberis aquifolium* and (d): *Hydrastis canadensis*.

RetTime :1.707 / Polarity: Positive / Event: 1 / Source: DUIS

Intensity



RetTime :1.820 / Polarity: Positive / Event: 1 / Source: DUIS

Intensity

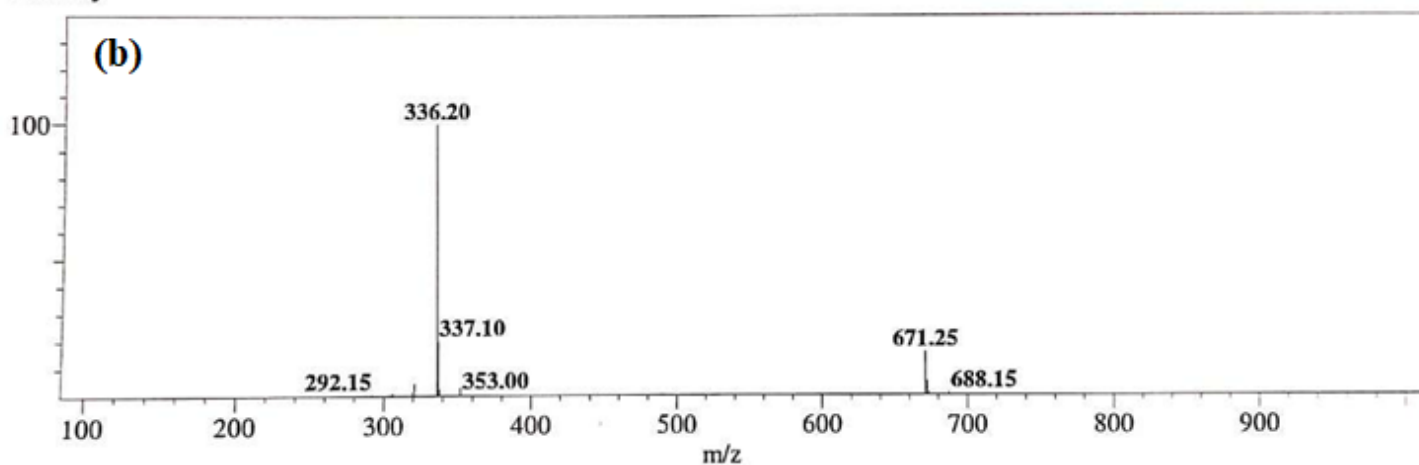


Figure 3

Mass spectra of berberine in (a): standard sample and extracted sample.

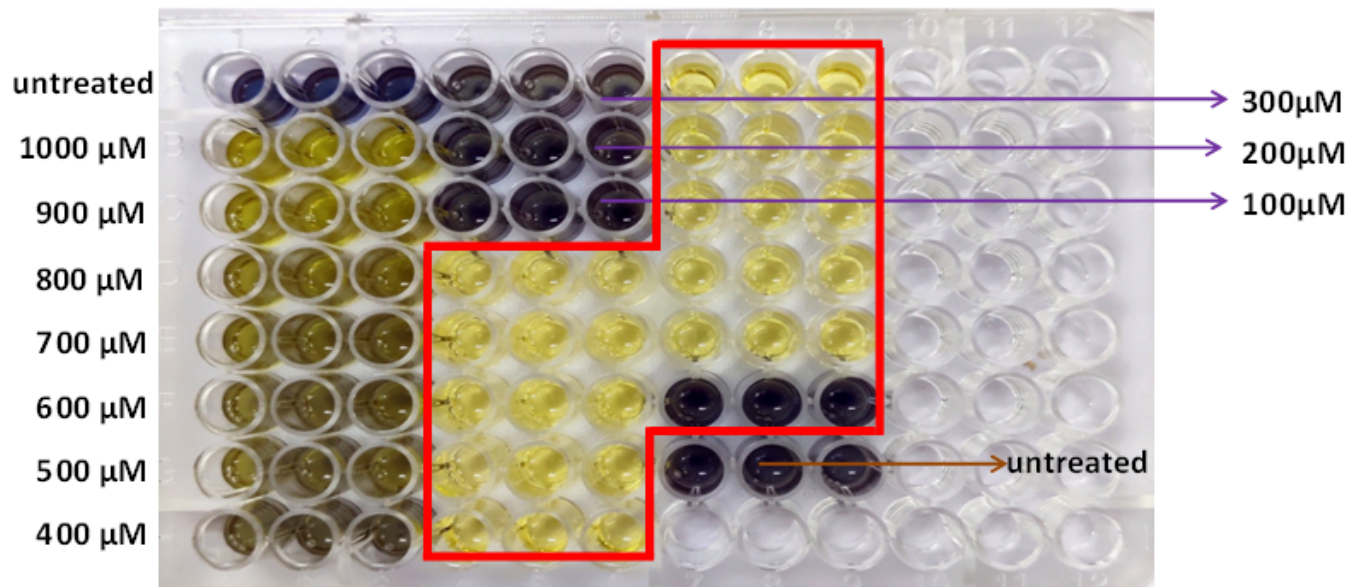


Figure 4

Treatment of berberine on *E. coli* with different concentrations (mentioned before respective wells) Red wells are Kanamycin treatment using same concentrations.

	Berberine	Kanamycin
LogIC₅₀	2.135	1.036
IC₅₀	136.3	10.87

IC₅₀ of Berberine on *E. coli*

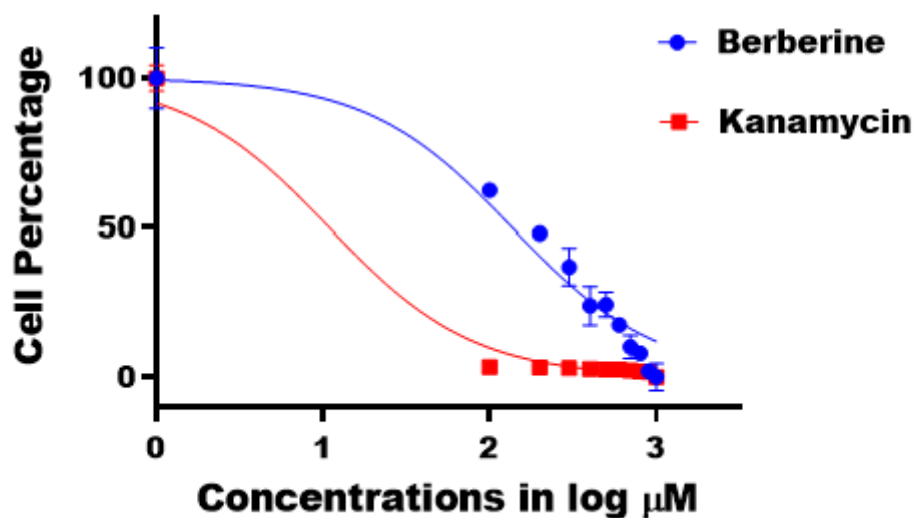


Figure 5

Graphical representation of IC₅₀ of berberine against E. coli and standard Kanamycin on E. coli with different concentrations.

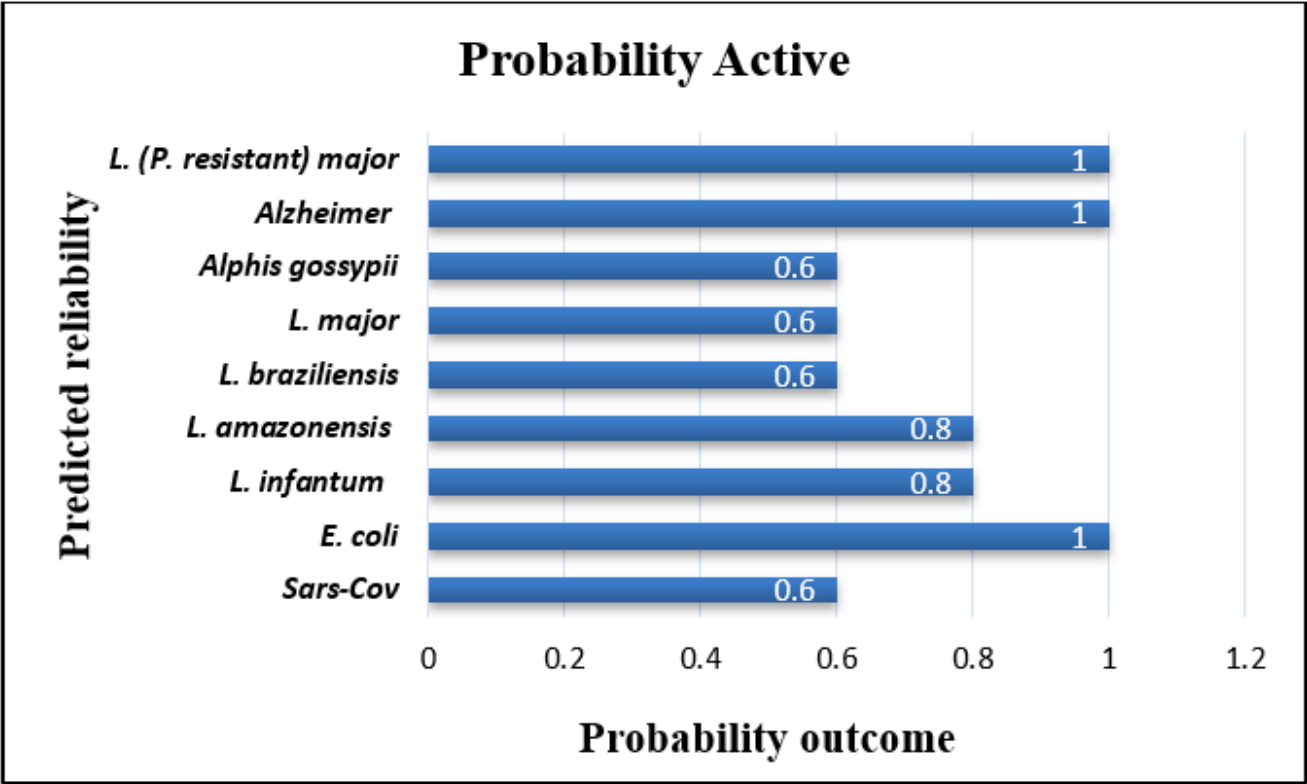


Figure 6

Artificial intelligence predicted activities of berberine.