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HPLC Fractioning to Study the Synergy and Antagonism of Rue Plant Seeds Alkaloid to Inhibit Topoisomerase II as Antitumor

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Abstract

DNA topoisomerases are ubiquitous, enzymes needed to control topological problems encountered during DNA replication, transcription, recombination and maintenance of genomic stability. They proved to be valuable targets for cancer chemotherapy. The seeds alkaloids extract of Rue plant (*Peganum harmala* L. Zygophyllaceae) were fractioned by high performance liquid chromatography as a suitable technique, HPLC waters company, gradient, Nova-Pak, C18*, 4µm, 3.9x150mm, mobile phase ACNwater pH10.7. Thirty-four separate solution depending on the retention time of eluents. The aim of this study is to investigate the trace compounds relatively and used as inhibitors for the topoisomerase II enzyme, the study the poison and suppressor model inhibitions and the synergy and antagonism activity of fractions. The visicinone, harmine, visicine and harmaline, were (2.86, 0.75, 13.14, and 16.62 mg/100mg of plant powder. The harmine >vicicinone> harmaline> vicicine as enzyme inhibitors. The unknown trace compounds gave the best inhibition relatively; the alkaloid fractions gave higher inhibitory units of fraction effective (FE). Results of seeds alkaloid fractions showed that the FE in state of fraction mixture was 36% inhibitory units, while summation of FE of each fraction alone gave higher level than their mixture, where sum of FE of them was 1229% inhibitory units. The suppressor inhibition model according to highest percent of the remain supercoiled DNA plasmid than production of linear and open nicked circular with high value in F8 (84%), the poisons inhibition model activity according to the highest percent of linear and nicked open circular DNA plasmid with high level in F31 (88%). There are a trace compounds within some fraction but that the effectiveness of inhibitory relatively higher than known compounds (harmine, vicisinone, harmaline, and vicisine), this can be purified and used as somewhat better inhibitors. There is antagonism activity among seed alkaloids fractions. The all fractions showed a poison and a suppressor model inhibition action in varies percentage, the suppressor inhibition appear in high value in F8 (84%), while the poisons inhibition model gave high level in F31 (88%).

Keywords: *P. harmala*, Poison and Suppressor inhibition, Side effect, Tumor target

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Introduction

Topoisomerase II protein is important to DNA replication, chromosome buildup and chromosome isolation. Inhibitors of topoisomerase II are essential medications as chemotherapy of numerous neoplasms including bosom growth, lung malignancy, testicular tumor, lymphomas and sarcomas. This Topoisomerase compound is one anticancer target [1]. Topoisomerase II breaks double-strand and passes doublestranded DNA through the nick to allow relaxation of supercoiled DNA [2]. They are essential for the survival of all eukaryotic organisms and as a target of essential antitumor drugs [1]. Medicinal plants play a vital role in a significant reduction of cancer treatment. It has been identified many of the herbs, which starts therapeutic effect by inhibiting hormones and enzymes of cancer, cause activation of DNA repair mechanism, anti-oxidant, production of protective enzymes, and increase the body's immunity [3]. Cancer is a genetic disease occurred by gene mutations associated with cell proliferation and cell death occurred by DNA damage [4]. In theory, the alterations at any step in the cell-killing pathway increase drug resistance of cancer cells, expensive anticancer drugs, and do not cure cancer and have serious adverse effects. Therefore, a new inhibition way for cancer, like DNA Topoisomerase II as a looking towards alternate and complementary medicine [5]. Plant secondary metabolites can be utilized as natural drugs to inhibition the activity of DNA topoisomerase, there is a need for the development of novel plant-derived natural drugs and their analogs, which may serve as appropriate inhibitors with respect to drug designing [6]. Wild Syrian rue (*Peganum harmala* L. Zygophyllaceae) seeds extract is one of the main components of an ethnobotanical preparation used in the treatment of neoplasms [7]. Its seeds, and other parts have been used as folk medicine, several researches has proved various pharmacological and therapeutic effects of *P. harmala* active alkaloids, especially harmine and harmaline [8]. Their devastating actions generate toxic effects of the patients, [9]. Synergistic and antagonistic drug interactions are important to consider when devel-oping mixtures of anticancer or other types of drugs.

Boik and Newman, and Boik *et al*., reported that the side effects of cancer drugs is a major problem in the treatment and the screening of all active compounds is very difficult, so researchers interested in finding the best treatments with less side effect, The more a few doses whenever the side effect was less [10, 11].Therefore, the main goal of this study to using the HPLC fractioning technique to investigation the action of Rue plant alkaloids, synergism and antagonism effect, getting trace alkaloids compounds that give best inhibition relatively easily. In addition, to study the model of topoisomerase II inhibition that conjugation with cell cycle levels.

Method and Material

Chemicals

Human Topoisomerase II, pHOT1 plasmid DNA, reaction solution material and buffers were purchased from TopoGEN CO., were provided in Chappell lab, Department of Pharmaceutical Science, college of Pharmacy, University of Kentucky, USA, they were stored at -70°C until use, harmaline. HCl, harmine. HCl, vicininone HCl, and visicine. HCl were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and dissolved in dimethyl-sulfoxide (DMSO) (Merck, Germany). They were stored at -20°C until use.

Plant material

The aerial parts of P. harmala were collected around Najaf province/Iraq. The plant was verified by Herbarium of Department of biology, Faculty of science, University of Kufa. The research done at University of Kentucky, college of Pharmacy, Department of Pharmaceutical Science (Jappell lab).

Preparation of extract

Rue plant seeds powder (Half kilogram) degraded by blander (fine powder), extracted with methanol (100%) by a Soxhlet apparatus 3000ml volume (10 cycles). Percolate by filter, evaporated by a rotary evaporator at a temperature of 45-50 Cº down to volume 1liter, 750ml of extracted solution was used to purify alkaloids by a separating funnel 1-liter volume, 2N HCl was added to obtain pH2, then, filtered and washed with 500ml chloroform three times. Adjust the pH to 10 by NH4OH. Also partitioned with 500ml chloroform three times. Chloroform layer was evaporated to dryness to obtain the alkaloid powder. The alkaloid fractioning by HPLC (waters company, gradient, Nova-Pak, C18*, 4µm, 3.9x150 mm, solvent of alkaloid was 100% methanol, mobile phase (ACN- water pH 10.7 by ammonia hydroxide), T1 and T2 of column were (37Cº), injection volume 10microliter,

Table 1.

Programmed flow rate by HPLC

No.	Time (min)	Flow rate l/min	%ACN	%H_2O and ammonia hydroxide (pH 10.7)
1	0	0.40	95.0	5.0
2	10	0.40	95.0	5.0
3	25	0.40	5.0	95.0
4	35	0.40	5.0	95.0
5	36	2.50	95.0	5.0
6	37	2.50	95.0	5.0

Protocol for Topoisomerase activity, modified topoisomerase II drug screening user manual kit

detector Diode-array 190-500nm at 1.2nm, actually presser 400-460 psi, and programmed of flow rate was gradient and listed below **Table 1**.

Preparation seed alkaloids fractioning solutions by HPLC

According to Chappell methods the alkaloids was fractioning by HPLC depending on elution times, 1minute for each fraction, injection volume 10µl of 5mg/ml of seed alkaloids extract. Number of fraction was 34. The conditions and parameters of HPLC that listed in **Table 1**. Flow rate was 0.4ml/min, and therefore, the volume of each fractions were 0.4ml, evaporated by gas nitrogen, re-dissolved by the same volume of 0.4ml of 24%DMSO with buffer. These represented a stock solution, diluting to (1:4), 10µl of each fractions were mixed in one tube to represent the mixture of alkaloid with the same concentration of compounds that present in fractions in order to study the synergy and antagonism by compared the fraction affect alone with their mixture. One microliter of each fractions or mixture solution added to the reaction solution of enzyme (10µl) to obtain a final diluted factor (1:49).

(TopoGEN).

Topoisomerase inhibition assay The final volume of reaction solution of enzyme was 10µl, which prepared by mixing of two parts of solution that prepared previously. Part 1 (7µl), AB buffer contains pHOT1 plasmid and inhibitor; 4.8µl of MiliQddH2O was added into 0.25 ml PCR tube. 0.7µl of A:B buffer (1:1) 10x to get 1x, 0.5µl of pHOT1 DNA plasmid, The contents above were mixed very carefully; it was make up to 7µl by adding one microliter of different concentration of seed alkaloids extracts and positive control. Part 2 (3µl), Prepare another AB buffer 1x with Topoisomerase II, each 3µl of buffer

contains 0.5 unit of enzyme added to each reaction solution. Then, mixed each parts together carefully, incubated by PCR for 30 min at 37Cº, lid temperature 105Cº. Then, put the PCR tube into ice container before the stopping step. The stopping procedure of enzyme activity was the same of incubation time assay above .

Cleaned inhibition compounds The inhibitors compound interfering with gel DNA documentation, therefore, solution reaction must be cleaned from it, the final reaction solution was 13µl, add the same volume of Chloroform: Isoamyl Alcohol (CIA) (24:1), vortex briefly, and then microfuge for 5sec. The blue colored (upper layer) was drown and load onto agarose gel.

Agarose gels and loading sample methods

7µl of reaction solution was added into none Ethidium Bromide (EB) agarose gel 1% directly, fresh Electrophoresis buffer (50x of TAE Gel buffer; 242g Tris base, 57.1 ml glacial acetic acid and 100 ml of 0.5 M EDTA), then the buffer was diluted to 1x to use for gel separation. In addition, the gel had 1x TAE buffer. 1.5-2.0 V/Cm until the dye front has traveled about 80-90%. Stained with EB (0.5µg/ml) for 20min, distained with water for 15 min, photo documentation by gel documentation (Bioneer).

Quantitative analysis

The quantitative analysis of pHOT1 DNA plasmid bands refers to the enzyme inhibition, which done by ImageJ 1.4 program.

The inhibition of topoisomerase II relaxation activity by each alkaloids fractions according to remain supercoiled DNA form by suppressor action and linear and open circular DNA forms by poisons action of alkaloids fractions calculated by the following modified equation 9 : Inhibitory action of fraction % (IAF %) = (S-S0)/ (Scontrol-S0) X 100

-Where S control is the percent of supercoiled DNA in the control (without enzyme and test compounds).
-S is a sum supercoiled, linear and open circular DNA amount, enzyme and test compound dissolved in 2.4%DMSO.
-S0 is a sum supercoiled, linear and open circular DNA amount, enzyme and DMSO only.
-S control is a sum supercoiled, linear and open circular DNA amount without enzyme, inhibitors, and DMSO.

The IC50 is define as the concentration of the test compound resulting in a 50% reduction of relaxed DNA.

Qualitative analyses of pHOT1 DNA plasmid bands

The qualitative analyses useful to detect the two kinds of Topoisomerase II agents: those that inhibit the activity of the enzyme (Catalytic Inhibitory Compounds CICs, sometime named as a suppressor compound) and those that stimulate formation of the cleavage complexes (Interfacial Poisons or IFPs, sometime

named as a poisons compounds). CICs may affect enzyme at any levels of cell cycle, such as blocking access to DNA and then decreasing the conversion of supercoiled of DNA. FIPs are another type of inhibitor that blocks the resealing step of the reaction on DNA such that open nicked DNA (OC. DNA) tend to accumulate according to TopoGEN.

Results and Discussion

Fractions of alkaloids as topoisomerase II inhibitors

The fractioning of alkaloid was accrued by High Performance Liquid Chromatography depending on time of fraction elution, Choosing the right mobile phase solvent in order not to affect the chemical composition of fraction solution, then interfering with enzyme inhibition activity, therefore, the mobile phase should be volatile and no toxic, in addition, the pH of mobile phase must be alkali by using ammonium hydroxide. The Rue plant alkaloids are perfect specialists to be utilized as novel antitumor medications with lesser side effects [12]. A several in vitro and in vivo researches have uncovered that these cytotoxicity and antitumor impacts of *P. harmala* are identified with its communication with RNA [13]. DNA and its synthesis [14, 15]. Mechanistic studies indicated that beta-carboline derivatives inhibit DNA topoisomerases II and interfere with DNA synthesis [16]. Beta-carboline alkaloids such as harmine are present in medicinal plants such as *P. harmala* that have been used as folk medicine in anticancer therapy. Harmine and harmaline implies a new approach in leukemia treatment [17]. In this study, there are thirty-four of seeds alkaloid fractions with determination of some alkaloid compounds weight (mg/100mg) of visicinone, harmine, visicine and harmaline, where were (2.86, 0.75, 13.14, and 16.62 mg/100mg of plant powder. Depending on the relative inhibition measured by the equation:

[Relative inhibition= inhibitory unit of compound/compound% in plant] The results show harmine>vicicinone > harmaline>vicicine, **Table 2**, this agree with Sobhani *et al*., where showed the order of potency was harmine > harmane > harmaline, [7. 18], and The alkaloid DNA binding affinity was ordered as harmine>harmalol>harmaline>harman e >tryptoline [18, 14]. This effect was attributed to the betacarboline content of the extract and potency of the alkaloids were determined as harmine >harmane >harmaline in a way that treatment with the total extract showed weaker inhibitory effect than treatment with every individual alkaloid [19]. Many authors have also reported intercalation of several carbolines into eukaryotic DNA [19, 20]. This interaction of beta carbolines cause significant structural changes in DNA and interfere with its synthesis [20, 14]. When analysis forms of DNA plasmid, the alkaloid extract were inhibiting Topo II catalytic activity

II (decrease in activity, sometimes called 'negative synergy). Antagonism may also occur, particularly in some interactions between orthodox medication and some herbal products [23].

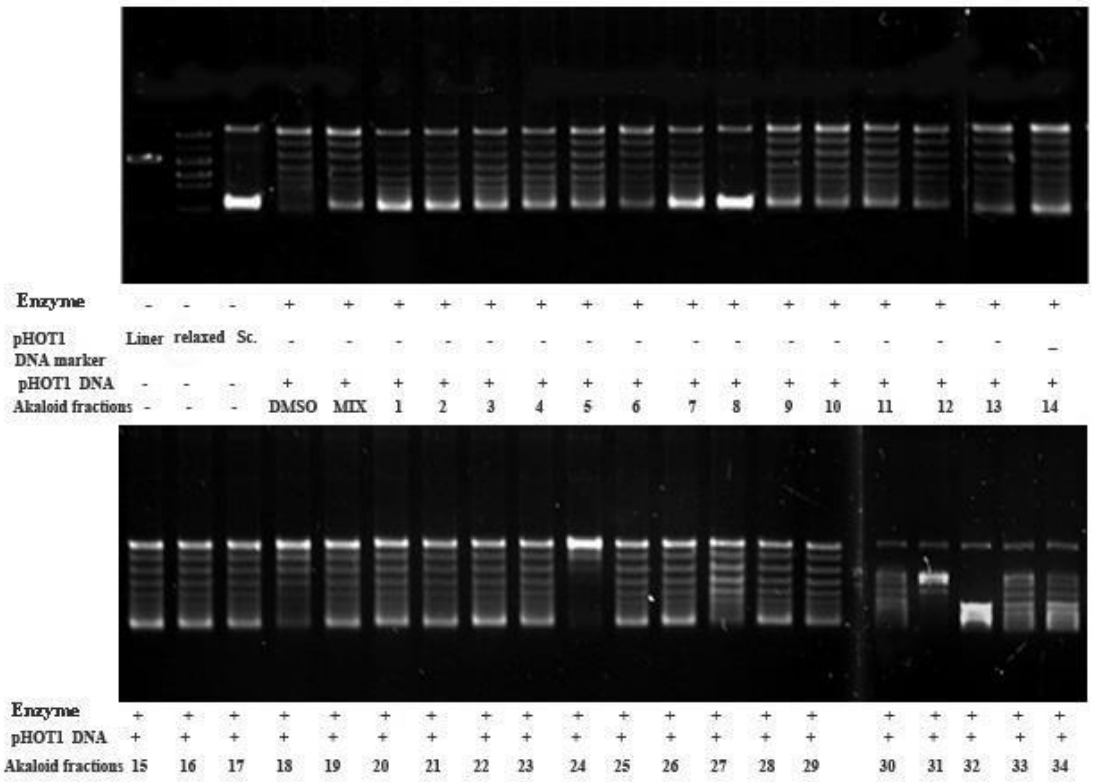


Figure 1.

Shows the non-ethidium bromide agarose gel of fractioning activity of Topoisomerase II.

(DNA relaxation) in both way (poisons and suppressors), by ability to trapping cleavage complexes and enzyme and/or plasmid respectively, therefore, these extracts act as poisons by blocking relegate of nicked strand and as suppressors by attacked enzyme or DNA plasmid **Figure 3.** The summation of suppresser and poison inhibition of each fraction fig. (2) between (16-94%) in F6 and F8 respectively. F30, F32 and F34 show activation action to the enzyme, the F1, F2, F3, F4, F7, F8, F9, F10, and F32 showed suppressor inhibition model according to highest percent of the remain supercoiled DNA plasmid than production of linear and open nicked circular with high value in F8 (84%), F6, F11, F13, F14, F15, F16, F17, F18, F16, F20, F21, F22, F23, F24, F27, F30, F31, F33, and F34 having poisons inhibition model activity rather than poison activity according to the highest percent of linear and nicked open circular DNA plasmid with high level in F31 (88%), **Figure 1., Table 2.** Synergy and antagonism study of seeds alkaloids fractions as a topoisomerase II inhibitors Synergy meaning of 'working together' or definition as 'an effect of a combination of substances appear more activity of compounds individually [17]. Berenbaum shows More precise definitions, incorporating In this study, the total activity (suppresser and poison activity) of fractions was had 36.0% inhibitory units, while sum of fraction effect of each fraction alone was higher level than mathematical considerations, who prefer use of isoboles in determining synergy, since these are concerned with the effect and not with the mechanisms involved [21]. Williamson apply Synergy as to herbal products [22]. The synergy and antagonism activities, and determination of the IC50 of enzyme inhibition of these compounds were impossible here, in addition to the unknown compound of fractions, therefore the study was focused only on measuring the fractions effect (FE) alone and compared with their mixture during survey of alkaloid contents. The HPLC was fractioning alkaloids of seeds with the same concentrations percent in rue seeds, therefore, the synergy and antagonism activities estimation depended on compared fraction effects of each fraction alone with the mixture, also, we depended of the HPLC quantitative analysis of some compounds to determine the relative inhibition activity. In this research, the HPLC technique can facilitate finding strengths and weaknesses of the plant as an inhibitor or an inducer of any as fast techniques without the separation and purification of the compound beforehand. Also to obtain the best fraction that give good enzyme inhibition relatively, the mixture of fractions appears having antagonism effect on Topoisomerase

their mix, where was 1229% inhibitory units **Figure 3**. The decreasing of fraction effect FE may have occurred by combination of alkaloids in their fraction together, and weaken the

formation of hydrogen bonds between the compounds and the plasmid and/or enzyme. This results agree with Cao *et al*., which reported that this effect was determined as harmine >harmane >harmaline in a way that treatment with the total extract showed weaker inhibitory effect than treatment with every individual alkaloid [19]. For further research, although the other fractions appeared less amount of compound content compared with harmine, vicicinone, harmaline, and vicicine compounds but higher relative inhibition activity **Figure 2, 4** and that refer to present trace compounds may be used as antitumor with high activity and less side effect. The research confirm attributed to the betacarboline content of the extract and potency of the alkaloids were

that separation technique is the easiest way to investigate the efficiency of the high relatively trace compounds, and can used in preparation for examination and identification of their and neglecting the rest fractions. In **Figure 3.** the percent of pHOT1 DNA forms after, the high percent of supercoiled remain refer to the high suppressor level activity of fraction enzyme, while other forms especially linear refer to poison model of enzyme inhibitions, anyway, the poison occurred after enzyme cleavage but cannot re-ligation of plasmid again.

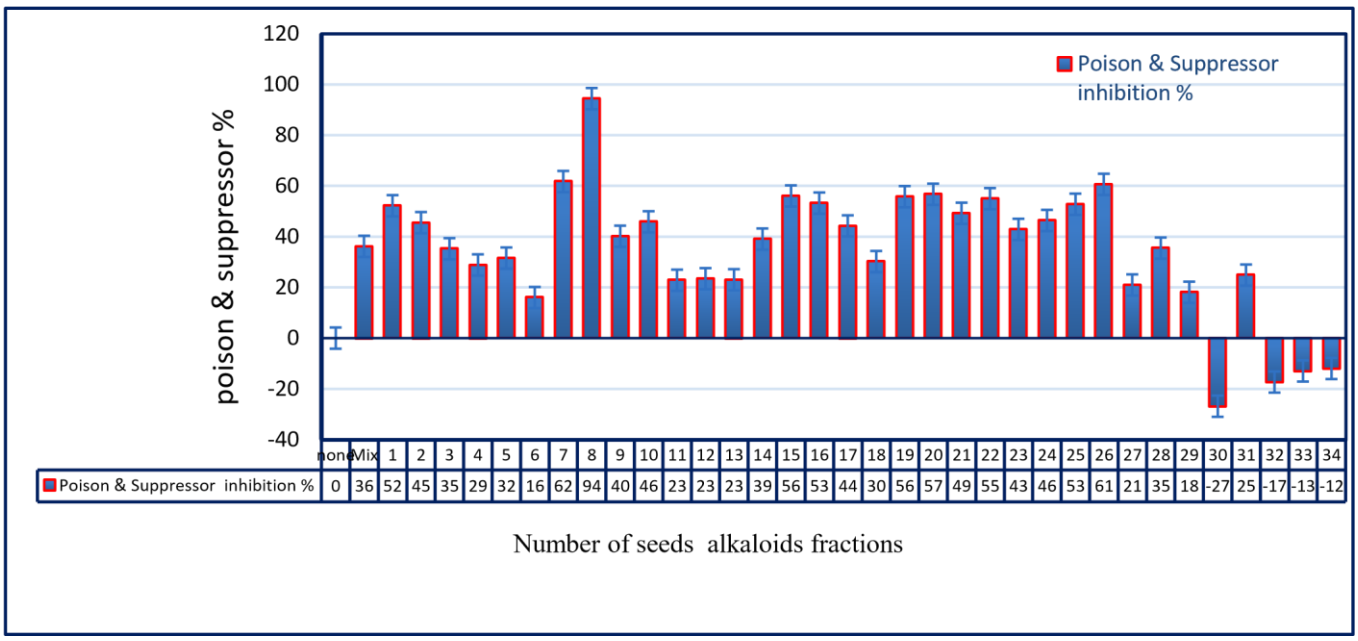
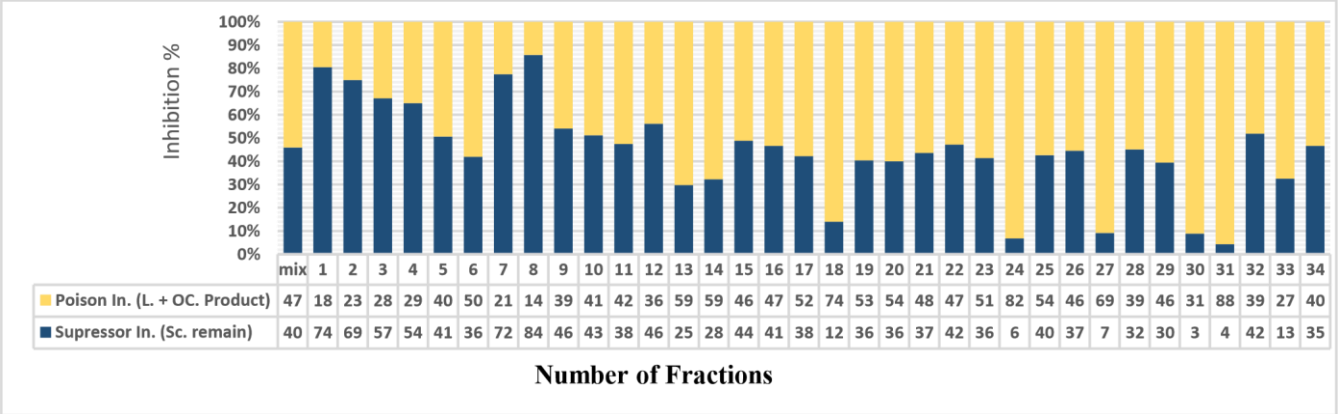


Figure 2.

Shows the relaxed inhibition and enhancing activity percentage activity of thirty-four alkaloid fractions of Seeds compared with their mixture.



	mix	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
Poison In. (L. + OC. Product)	47	18	23	28	29	40	50	21	14	39	41	42	36	59	59	46	47	52	74	53	54	48	47	51	82	54	46	69	39	46	31	88	39	27	40
Supressor In. (Sc. remain)	40	74	69	57	54	41	36	72	84	46	43	38	46	25	28	44	41	38	12	36	36	37	42	36	6	40	37	7	32	30	3	4	42	13	35

Figure 3.

Shows the suppressor and poisons model according to supercoiled, summation of linear, and open nicked circular forms percentage of thirty-four alkaloid fractions of Seeds compared with their mixture.

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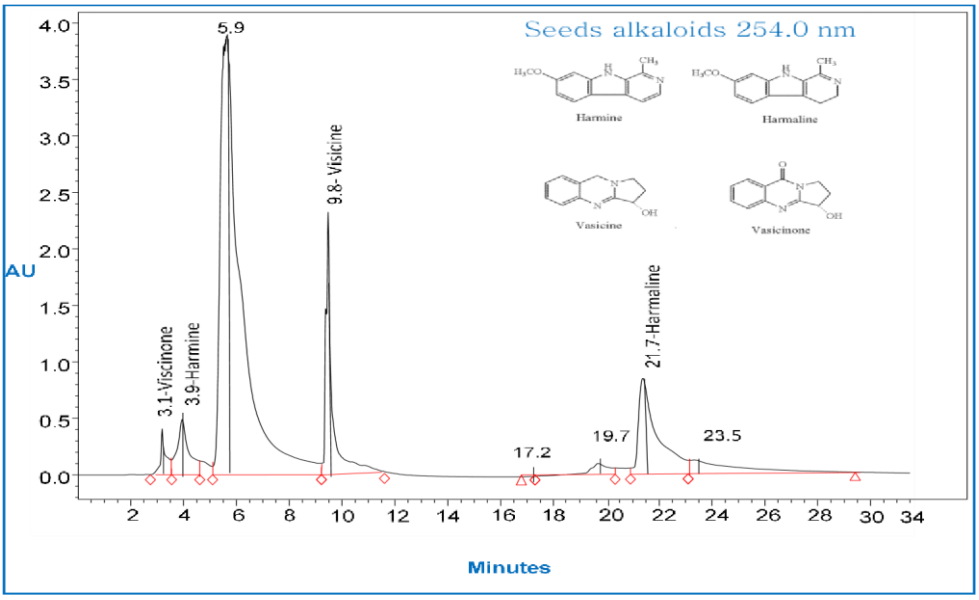

Figure 4.

HPLC of Seeds alkaloids of *P. harmala*. absorbance traces at 254.0 nm.

Table 2.

HPLC Parameters of seed alkaloids Fractions of Rue plant 254nm

NO.	Fractions	Com.	Retention Time Rt.(min)	Peak Area (µV*sec) of seeds	Weight mg/100mg alkaloid	Inhibitory units	Relatively inhibition (inhibition unit/compound% in plant)
1	F1	-	-	-	-		
2	F2	-	-	-	-		
3	F3	Visicinone	3.1	2549336	2.86		12.23
4	F4-F5	Harmine	3.9	18418624	0.75		81.33
5	F5-F6	-	-	-			
6	F6-F9	-	5.9	135177211	-		
7	F10	Visicine	9.8	30125985	13.14		3.50
8	F11	-	-	-	-		
9	F12	-	-	-	-		
10	F13	-	-	-	-		
11	F14	-	-	-	-		
12	F15	-	-	-	-		

13	F16	-	-	-	-		
14	F17	-	-	-	-		
15	F18	-	17.2	23653	-		
16	F19	-	-	-	-		
17	F20	-	19.7	5480877	-		
18	F21	-	-	-	-		
19	F22-23	Harmaline	21.7	199280854	16.62		4.5
20	F24	-	23.5	12365337	-		
21	F25	-	-	-	-		
22	F26	-	-	-	-		
23	F27	-	-	-	-		
24	F28	-	-	-	-		
25	F29	-	-	-	-		
26	F30-32	-	30.0	12657	-		
27	F33	-	-	-	-		
28	F34	-	-	-	-		

Competing interests

Authors declare that we have no competing interests.

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