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Identification and Characterization of Bioactive Compounds from *Ruta graveolens* L. leaf Extract against *Najanaja* Venom.

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ABSTRACT

One of the serious health problems left unnoticed is snake envenomation. There are various synthetic antivenin available in market which has various side effects. Thus the aim of the study is to use the leaf extracts of *Ruta graveolens* against *Najanaja* venom by *in vitro* methods and characterize the bioactive compounds present inthe extracts of*Ruta graveolens*.In all the i*n vitro* studies such as procoagulant, phospholipase, fibrinolytic and cholinesterase activity had greater inhibition activity against venom was observed. Gas Chromatography analysis of the extracts showed the presence of compounds, 4 – Nerolidylcatechol, Bergapten and Beta – sitosterol which indicates that *R. graveolens* is a potential candidate against envenomation.

Keywords: Envenomation, *Ruta graveolens*, *Najanaja*, Bergapten.

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INTRODUCTION

Snakebite is a major health problem that is faced in day to day life in rural and hill region that is being left unnoticed. About 5million human are estimated to suffer from snake bite every year worldwide of which 1,25,000 deaths occurs[1-3]. In India, the most common poisonous snake species found are cobra (*Najanaja*), krait (*Bungaruscaeruleus*), Russell's viper (*Daboiarusselli*) and saw-scaled viper (*Echiscarinatus*). The first two belong to the family of Elapidae and the next two belong to the family of Viperidae [4-6]. Snake venom is combinations of complex organic mixtures. They are made up of proteins, toxins and enzymes that assist them in capturing prey and digesting them. Majority of the snake venom constitutes enzymes and toxins – Cardiotoxin, Neurotoxin and Phospholipase A2 which are responsible for various toxic effects on affected victims such as heart failure, amputations, deformations and renal failure. Snake venom also induces blood clotting, hemorrhage, necrosis, proteolysis and several other effects [7-10]. The venom of *Najanaja*is a neurotoxin which affects the central nervous system of the victim thus leading to paralysis and death [11-13].

The antivenom immunotherapy is the available treatment for snake envenomation in the recent scenario. It is the present effective method against envenomation but it has its own fallouts. They are as follows

- Side effects which causes anaphylactic shock, pyrogen reaction and serum sickness.
- Failure to neutralize, leading to tissue damage.
- Geographical variation in venom composition of snakes [14-15].

In Ayurvedic system many types of plant species were reported to have antivenom activity. The traditional method of producing antidote is followed all around the world. Plants are thus consist to have the ability to inhibit or neutralize the action of snake venom and so making plant a potential for producing antidote for snakebites [16-23]. Also plant derived inhibitors that are been isolated are reported to possess antivenom activity [24-25].

Ruta graveolens L. (Rue) is an herbaceous perennial plant belonging to the Mediterranean region. In many parts of the world the plant species is been cultivated.Rue has been introduced into various part of the world due to its cultural value and use in traditional medicines for the relief of pain, eye problems, rheumatism and dermatitis. *R. graveolens* have shown antibacterial, analgesic, anti-inflammatory, antidiabetic and insecticidal activities [26-30].Rutin and quercetin are the main active flavonoids present in *R. graveolens* and its volatile compound has higher quantity of aliphatic acids, alcohols and ketons[31, 33]. In the present study the active compounds from *R. graveolens* leaf are to identified and characterized against *Najanaja*venom.

MATERIALS AND METHODS

Collection of snake venom

The venom of *Najanaja* was collected from the Green cross corporation, Erode branch, Tamil Nadu, India and stored in deep freezer until use. The collected venom was diluted in saline (pH 7.2-7.4) with concentration of 5mg/ml in ratio of 1:100 as stock. Further 1:500 dilution of stock solution was made and it was used as working concentration. The protein concentration in snake venom was estimated by Lowry *et al* [34] method.

Collection of leaf sample

The leaves were collected from the plant *R. graveolens* and it was shade dried till the moisture was removed completely. Then the leaves were grinded finely and stored at room temperature.

Preparation of extract

The leaf extract was prepared by adding 5 g of powdered leaf in 50 ml of solvent. It was left undisturbed for 72 hours. Then the extract was poured,air dried and collected from petri plate for further analysis.

***In vitro* analysis**

Procoagulant activity

The procoagulant activity was done according to the method described by Theakston and Reid (1983) [35] modified by Laing *et al*(1992) [36]. In brief, various volume of venom diluted in 100 µl PBS (pH 7.2) was added to citrated human blood plasma at 37°C. Coagulation time was recorded and the minimum coagulant dose (MCD) was determined as the venom dose, which induced clotting of blood plasma within 60 sec. Plasma incubated with PBS alone served as control. In neutralization assay, constant amount of venom was mixed with various dilutions of *R. graveolens* extracts. The mixtures were incubated for 30 min at 37°C. Then 0.1 ml of mixture was added to 0.3 ml of citrated plasma and the clotting time was recorded. In control tubes plasma was incubated with either venom alone or plant extracts alone. Neutralization was expressed as effective dose (ED), defined as the ratio ml antivenom (plant extracts)/mg venom at which the clotting time increased three times when compared with clotting time of plasma incubated with two MCD of venom alone.

Phospholipase activity

Phospholipase A2 activity was measured using an indirect hemolytic assay on agarose-erythrocyte-egg yolk gel plate by the method described by Gutierrez *et al.* (1988) [37]. Increasing doses of venom (µg) was added to 3 mm wells in agarose gels (0.8% in PBS, pH 8.1) containing 1.2% human erythrocytes, 1.2% egg yolk as a source of lecithin and 10 mM $CaCl_2$. Slides were incubated at 37°C overnight and the diameters of the haemolytic halos were measured. Saline was used as control. The minimum indirect hemolytic dose (MIHD) corresponds to the dosage of venom, which produced a hemolytic halo of 11 mm diameter. The efficacy of *R. graveolens* extract in neutralizing the phospholipase activity was carried out by mixing MIHD with different amount of plant extract (10µl - 50µl) and incubated for 30 min at 37°C. Then, aliquots of 10 µl of the mixtures were added to the wells in agarose-egg yolk-sheep erythrocyte gels. Venom without plant extracts were used as control. Plates were incubated at 37°C for 20 hours. Neutralization expressed as the ratio mg antibodies/mg venom able to reduce by 50% the diameter of the hemolytic halo when compared to the effect induced by venom alone.

Fibrinogenolytic activity

The fibrinogenolytic activity of *Najanaja*venom was determined by modifying the method of Ouyang and Teng (1976) [38]. Inhibition of fibrinogen was observed in SDS-PAGE (15%) according to the method described by Laemmeli (1970) [39]. In reaction mixture, bovine fibrinogen (20 µg) and venom (20 µg) in 5 mm Tris-HCl buffer (pH 7.4) and 10 mm NaCl were added together and was incubated for 15 minutes at 37ºC. For inhibition studies snake venom was pre-incubated with different volume of extract (10 µl – 50 µl) and then it was incubated with above mixture for 15 minutes at 37ºC. After 15 minutes the reaction was terminated by adding equal volume of sample buffer containing 0.2M Tris-HCl (pH 6.8), 20% glycerol, 10% sodium dodecyl sulfate (SDS), 0.05% bromophenol blue and 10 mM β-mercaptoethanol, followed by boiling at 100 ºC for 3 minutes.

GC MS analysis

GC MS analysis was done for acetone extract and ethanol extract using Thermo GC - Trace Ultra Ver: 5.0, Thermo Ms Dsq II. Column used was ZB 5 - MS Capillary Standard Non - Polar Column of dimension 30 Mts, ID : 0.25 mm, Film : 0.25 µm,Career gas used He, Flow : 1.0 ml/min temperature progress oven temp 70°C raised to 260°C at 6°C /min from SITRA, Coimbatore, TamilNadu, India.

RESULTS AND DISCUSSION

Procoagulant Activity

The protein content was quantified as 393.5µg/µl in diluted venom. In procoagulant activity, 0.395 mg of venom was required to produce the minimum coagulant dose (MCD) as shown in Table 1.Acetone extract and ethanol extract showed inhibition against procoagulant activity with 1µl of snake venom.40µlof acetone extract and 30µl of ethanol extractwere required to completely inhibit minimum coagulant dose of venomat

thrice the MCD coagulation (Table 2). In comparison with our results, higher amount of extract of *Andrographispaniculata*(1.8mg) and *Aristolochiaindica*(1.1mg) was required to inhibit 120 µgof russell viper venom [40]. The *M. pudica*plant extract (1.4 mg) were able to completely neutralize coagulant activity of 60µg of venom [41] which has comparatively lower inhibition activity than the plant *R. graveolens*.

Volume of snake venom (µl)	Volume of blood plasma (µl)	Coagulation Time (Sec)
1	300	59.4 ± 1.17
2	300	40.6 ±1.2
3	300	28.6 ±1.40
4	300	16 ±1.41
5	300	8 ±1.06

Table 1: Determination of Minimum Coagulant Dose (MCD) for *Naja naja* venom

Volume of venom (µl)	Volume of Extract (µl)	Coagulation time (Sec)	
		Acetone extract	Ethanol extract
1	10	60.8 ± 1.76	119.8 ± 1.45
1	20	99.2 ± 1.62	155.6 ± 1.56
1	30	123.6 ± 2.70	180.6 ± 0.76
1	40	181.4 ± 1.33	242.2 ± 0.98
1	50	330.2 ± 1.75	363.2 ± 1.74

Table 2: Inhibition of Procoagulant activity by leaf extracts of *Ruta graveolens*.

Phospholipase Activity

The phospholipase activity of venom was calculated by measuring the hemolytic halo of 11mm diameter in agarose gel and that venom concentration is called as minimum indirect hemolytic dose (MIHD). In Table 3, the concentration of venom was calculated to be 5 µl (1.967 mg), which produced 10.96 mm halo in agarose gel. The inhibition of phospholipase activity of venom can be calculated from the concentration of plant extract at which the hemolytic halo (11 mm) was reduced to half of its diameter (5.5 mm).The hemolytic halo of 4.96 mm and 4.98 mm was observed to inhibit 5 µl (1.976 mg) of venom by 2.50 mg (50 µl) and 4.16 mg (45 µl) of acetone extract and ethanol extract respectively (Table 4). In contrast to our results, the amount of Tea extract required for inhibiting 17.1 µg of*Calloselasmarhodostoma* and 3.4 µg of *Najanaja*was 101 µg and 303 µg respectively [42]. Similarly 0.13 mg and 0.16 mg of *M.pudica*extract can inhibit 15 µg of cobra venom and 10 µg of krait venom respectively [41].

Volume of venom (µl)	Formation of hemolytic halo (mm)
1	2.62 ± 0.21
2	4.8 ± 0.17
3	7.42 ± 0.41
4	8.8 ± 0.31
5	10.96 ± 0.26

Table 3: Determination of MIHD value for *Naja naja* venom.

Volume of Venom (µl)	Volume of Extract (µl)	Hemolyyic Halo (mm)	
		Acetone extract	Ethanol extract
5	30	10.86 ± 0.11	10.58 ± 0.15
5	35	10 ± 0.1	8.92 ± 0.20
5	40	8.72 ± 0.15	6.96 ± 0.12
5	45	6.88 ± 0.19	4.98 ± 0.13
5	50	4.96 ± 0.11	4.3 ± 0.15

Table 4: Inhibition of Phospholipase activity by *Ruta graveolens* leaf extracts.

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Fibrinogenolytic activity

The inhibition of fibrinogenolytic activity of *R. graveolens* extract on snake venom was carried out and observed in SDS – PAGE. The ethanol extract and acetone extract of *R.* graveolensshowed solvent effect in SDS-PAGE during analysis offibrinogenolytic activity.Thus ethanol extract and acetone extract were left to dry and then the dried extractswere again dissolved with water to avoid solvent effect. In ethanol extract, venom and extract were added in the ratio of 1:2 (30 µl: 60 µl) and for acetone extract, venom and extract were added in the ratio 1: 3 (30 µl: 90 µl). Fig 1 and Fig 2 shows Fibrinogenolytic activity of ethanol and acetone extract using SDS – PAGE. It showed that 4.62 mg of acetone extract and 5.24 mg of ethanol extract were required to inhibit 11.805 mg of venom. Similar to our results,fresh leaf extract of *Camellia sinensis* and EDTA containing 22.5 µg and 46.5 µg respectively inhibits 16.9 µg of *Calloselasmarhodostoma*and *Najanaja*venom [42].

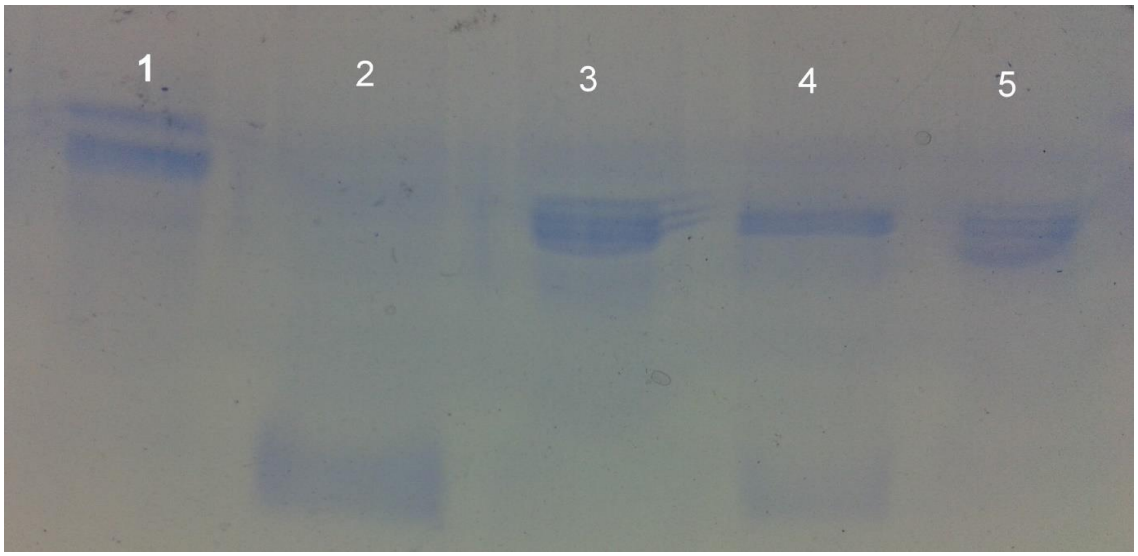

Figure 1: Fibrinogenolytic activity of Ethanol extract using SDS-PAGE.

Lane 1: BSA, Lane 2: Venom, Lane 3: bovine fibrinogen, Lane 4: Venom & fibrinogen, Lane 5: Venom, fibrinogen and ethanol extract.

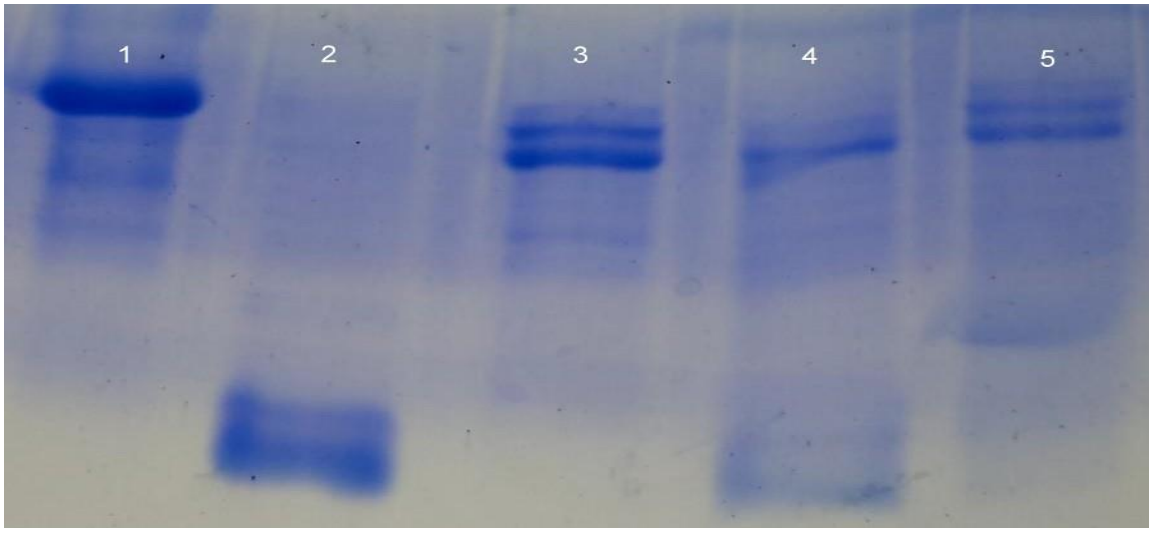

Figure 2: Fibrinogenolytic activity of acetone extract using SDS-PAGE.

Lane 1: BSA, Lane 2: Venom, Lane 3: bovine fibrinogen, Lane 4: Venom & fibrinogen, Lane 5: Venom, fibrinogen and acetone extract.

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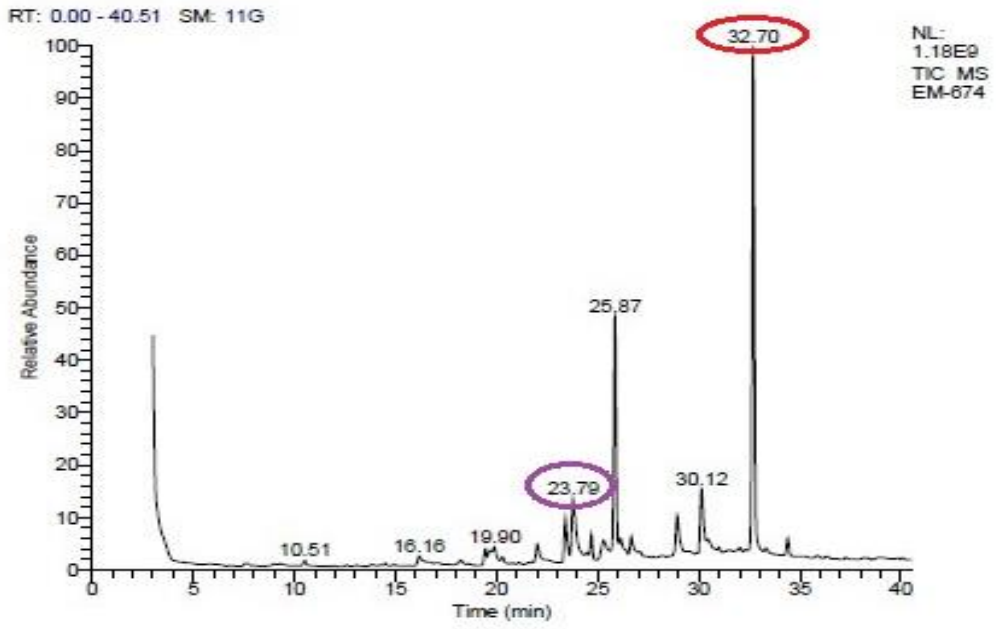

Figure 3: Graph representing the GC MS analysis of acetone extract

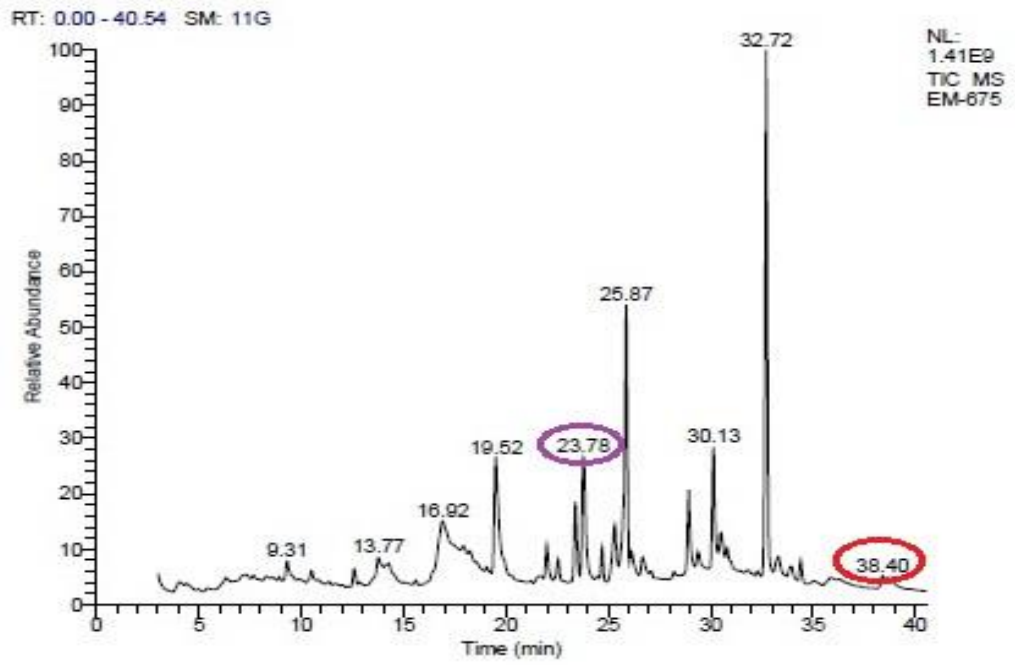

Figure 4: Graph representing the GC MS analysis of ethanol extract

GC MS Analysis of Leaf extract

The identification of active compound in the ethanol and acetone extract of *R. graveolens* were analysed by GC MS (Fig 3 & Fig 4). GC MS analysis of acetone extract and ethanol extract of *R. graveolens* showed the presence of various compounds. The Chromatograph of acetone extract showed the presence of compound 4 – Nerolidylcatechol and Bergaptin in the peak value of 32.70 and 23.79 respectively (Fig 3). The Ethanol extract chromatograph (Fig 4) has shown the peak value 23.78 and 38.40 for Bergaptin and Beta – sitosterol respectively. The said compounds such as4 – Nerolidylcatechol, Bergaptin and Beta – sitosterolwhich

were reported to possess antivenom property which was already reported [32].Rutin, a major compound present in the plant *R. graveolens*has also been reported to have antivenom property [31].

CONCLUSION

Snakebite, now a days is a major health hazard that causes high death rate especially in India and there are many medicinal plants that are recommended for snake bite. Scientific attention has been given for last 20 years for using medicinal plant in snake envenomation. Antisnake venom, usually derived from horse sera remains as antidote for envenomation. They contain horse immunoglobulin which frequently causes complement mediated side effects. Other proteins in the sera cause serum sickness and occasionally, anaphylactic shock [14]. The WHO estimated 80% people rely on herbal medicine [7].

Various pharmacological activities such as procoagulant activity, phospholipase activity, caused by *Najanaja*venom were been carried out. Neutralization of the pharmacological activity was carried out using *Ruta graveolens*. The results showed that *Ruta graveolens* was capable of neutralizing coagulation of blood. In phospholipase A2 activity the plant extract *Ruta graveolens* was able to reduce the halos to half the diameter of the control. Fibrinogenolytic activity also showed inhibition of venom. GC MS analysis of both acetone and ethanol extract showed the presence of the compounds 4 – Nerolidylcatechol, Bergaptin and Beta – sitosterol which were already reported to have antivenom property.Thus it is concluded that the plant extract of *R. graveolens* has the high neutralizing potency against snake venom and the confirmation was availed by the presence of antivenom compounds in the plant extract. Studies by *in vivo* methods can give further confirmation on the antivenom potency of *R. graveolens* leaf extract. Thus by the use of medicinal plants the side effects of the antivenom serum can be overcome and also it can pave a new way for treating snake envenomation.

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