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RESEARCH ARTICLE

ASSESSMENT OF SNAKE ANTIVENOM ACTIVITY OF TAMARINDUS INDICA SEED COAT EXTRACT

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Abstract

Snakebite is a life-threatening medical emergency, and globally responsible for millions of deaths. In snakebites accidents only deaths are not a concern, it leads to more morbidities. Due to scanty healthcare facilities in rural areas of India, many people seek alternative treatment available in ethnic practices. *Tamarindus Indica* (TI) plant is rich in medicinal value and used to treat many diseases including snakebite treatment traditionally. In view of this TI seed coat extract (TISCE) was evaluated for antivenom activity. The phytochemical screening of TISCE was performed to understand its chemical composition. TISCE was evaluated for antivenom activity against Indian cobra venom (ICV), common krait venom (CKV), Russell's viper venom (RVV), and saw-scaled viper venom (SCV) for phospholipase A2 (PLA-2), haemorrhagic *in vitro* and *in vivo*, procoagulant, proteolytic activity, and lethality studies. TISCE majorly contains saponins, glycosides, alkaloids, and phenolic compounds. Minimum indirect haemorrhagic dose (MIHD) observed for ICV (12.5 µg), CKV (5.0 µg),RVV (10.0 µg), and SVV (12.5 µg). TISCE inhibits the procoagulant activity of all venoms at a concentration of 18.0 µg. It also shows the neutralization of proteolytic enzymes of venom in a dose-dependent manner. A pre-incubated mixture containing five lethal dose 50 (LD_{50}) of venom and TISCE was injected intravenously, all mice survived as venom neutralized by TISCE. The present study demonstrates the ability of TISCE to neutralize snake venom using suitable *in vivo* and *in vitro* methods. Further studies required to unravelling the specific active chemical constituent of TISCE that may used as novel alternative snakebite treatment. TISCE was able to prolong the deaths during the simulation study and may be used in the topical pharmaceutical formulation that will reduce local venom reactions causing much morbidity, which will collectively with Anti-snake venom (ASV), used to treat envenomed patients more effectively.

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Introduction:-

Snake is a mysterious creature with many myths and most animus in the universe. A snake has a natural power to prepare unique fluid called venom containing numerous toxins, and enzymes mainly developed to kill the prey and protection from predators. Snakebite is a global disaster influencing about 4.5 - 5.4 million incidences, resulting in 1.8 - 2.7 million envenomation's, and 81,000 to 138,000 deaths. Most of the deaths occur in tropical and subtropical parts of the world, which includes developing or underdeveloped countries, where health management systems are underprivileged. In India solitary, estimated that 2.8 million snakebites occur, resulting in 46900 deaths annually. In rural areas, snakebite is an occupational hazard and causes death amongst farmers, catchers, and forest workers as they have more interactions in fields so it has a major social impact on the family losing an earning person (Gupta 2014). Considering the global impact of snakebite deaths, and to achieve effective control on it, the World Health Organization (WHO) included it in a Neglected Tropical Diseases.

In the world, around 3709 species of snakes observed, out of which only 700 venomous, mostly belongs to the family Elapidae, Viperidae, Atractaspididae, Colubridae, and Hydrophidae. In India, around 220 types of snakes observed, which includes 60 venomous species out of that four snakes are medically important namely Indian cobra, Common krait, Russell's viper, and Saw-scaled viper (WHO 2010, Singh and Singh 2013).

Snakebite leads to destructive physiological effect on the human body functions, and parts because the venom toxin targets almost all parts of the body, and its function. Nerve toxins cause lethal paralysis, which may cause death by slow suffocation. Some enzymes distort normal blood clotting, and lead to catastrophic haemorrhage, while deadly blood clot affects the heart, brain, and lungs. Venom proteins that demolish skin, muscle, other tissue causing dreadful destruction of hand, arm, and leg tissues. Some venom proteins affect vital organs like the kidney, heart, lungs, nervous system causing local, and generalized pain (Bawaskar and Bawaskar 2019). ASV is the only backbone of treatment containing equine immunoglobulin's prepared by immunizing equines with the venom. In India, polyvalent ASV is manufactured, which effectively shows the neutralization of ICV, CKV, RVV, and SCV (Wachtel and Cole 1964).

India has a very old ancient culture, ethnic practices, and herbal folk medicines in the form of plant sap, paste, decoctions, powders, and pills which are used for the treatment of many diseases including snakebite (Thirunarayanan 2013). Present immunotherapy used for snakebite treatment has some side effects, the cost of treatment is high which is not affordable to people from tropical, and subtropical countries like India. Many researchers are trying to develop a novel antidote against snakebite, which will be cost-effective, easily manufactured, and have minimal side effects. Considering all reviews, and data published, the herbal antidotes can be a promising alternative (Upasani et al. 2017). Many medicinal plants are extensively used by ethnic groups in India to treat snakebites (Nadkarni 1976, Kapoor 2005,Dey and De 2012, Gupta 2014). TI is one of the most reviewed plants, like a snake antidote by ethnic communities in India, Sudan, Nigeria, Bangladesh, and most of the tropical countries.

The current study was undertaken to evaluate the potential of TISCE on neutralization of venoms of medically important snakes of India (Vishwanath, Gowda, and Girish 2006, Al-Ani et al. 2017, Sailakshmi and Rao 2012, Biomedicine 2012). TI is one of the most important multipurpose tropical fruit tree in the Indian subcontinent belong to the monotypic genus. It has a broad spectrum of activities like antimicrobial, antioxidant, laxative, abdominal pain, wound healing, antidiabetic, cellular system, antiasthmatic activity, and anti-inflammatory (Isha, Milind, and Division 2012, Sudjaroen, Haubner, and Wu 2005). Seeds of TI reported to contain phenolic antioxidants, mainly oligomeric procyanidins, epicatechin, taxifolin, and eriodyctiol, crude fibers, tannins, reducing sugars, saponin glycosides, and alkaloids (Rana and Sharma 2018, Kazi et al. 2008). It was observed that most of the active compounds present in the seed coat, considering this seed coat extract taken for further study.

The presence of antivenom activity in TISCE was evaluated against venoms of medically important snake's for inhibition of PLA-2 enzymes, neutralization of haemorrhagic activity, neutralization of procoagulant activity, protease inhibition, and neutralization of lethality. TISCE shows effective antivenom activity against ICV, CKV, RVV, and SCV. TISCE shows neutralization of haemorrhagic activity in a dose-dependent manner, it also shows the neutralization of phospholipase enzyme activity, haemorrhagic activity, procoagulant activity, and protease activity. TISCE completely neutralizes five LD_{50} of venoms, and the present study shows that TISCE contains certain chemical constituents, which have the ability to neutralize ICV, CKV, RVV, and SCV toxins.

Material and Methods:-

Materials

The seeds of Tamarindus Indica L. (Fabaceae) were collected from Manakarnika Aushadhalaya, Pune, Maharashtra, India. Agharkar Research Institute, Pune, authenticated the sample by report no. AUTH 21-03. The local name of the plant is Tamarind (Imali). Haffkine Bio-Pharmaceutical Corporation Ltd., Pune, Maharashtra, India provided ICV, CKV, RVV, and SCV. Healthy Swiss albino mice weighing about 18 - 22 g were used for the study. The animals were housed in well-ventilated cages, maintained under standard conditions with *ad libitum* feed, and water. Agarose purchased from Sisco research laboratories Pvt. Ltd, Maharashtra and Bovine serum albumin from HiMedia laboratories Pvt. Ltd, Maharashtra. All chemicals used were of analytical grade.

Methods:-

Extraction of TISCE

Seed coat was removed from the white kernel, and ground into a fine powder. The fine powder extracted with ethanol 99.0 % using the soxhlet apparatus at 68°C for 5 hours (hrs), and allowed to evaporate the ethanol in vacuum oven. The crude extract was stored in an air-tight container at room temperature (Vishwanath et al. 2006).

Phytochemical analysis of TISCE

Phytochemical analysis of TISCE performed as per the test procedures mentioned in the book of Khandelwal K., (2017).

Toxicity studies of TISCE

To study the toxicity of TISCE, a group of six mice injected with different concentrations of TISCE, and one group served as a control injected with normal saline. The mice observed for toxicity signs like excitability, dullness, in appetence, and weight gain for a period of 7 days.

Snake venom characterization

Protein determination

The protein content of ICV, CKV, RVV, and SCV was determined by the biuret method (Layne 1957).

Venom LD_{50} assay

To determine LD_{50} of venoms a range-finding study performed to narrow the range, and graded concentrations of ICV, CKV, RVV, and SCV are prepared separately in normal saline, and injected into a group of six Swiss albino mice weighing 18 - 20 g intravenously. The deaths were recorded after 24 hrs and LD_{50} is calculated by the Reed-Muench method (Saganuwan 2016, Okoroma et al. 2012, Yi et al. 2015, Harshbarger 2014, Pawade 2016).

Neutralization of venom haemorrhagic activity (*In vitro*)

The agarose–erythrocyte–egg yolk plate method performed according to Gutierrez et al., (1987) with some modifications used to observe minimum haemorrhagic dose (MHD) of venoms. The agarose–erythrocyte–egg yolk plate was prepared using 1.2% horse erythrocytes, egg yolk, and agarose in phosphate buffer (pH 8.1). ICV, CKV, RVV, and SCV concentrations (0.1-0.6 µg/µl) was added to 3 mm wells, and plates were incubated at 37°C for 20 hrs, and the diameters of the haemolytic haloes were measured. The MIHD corresponds to a dosage of venom, which produced a haemolytic halo of 11 mm diameter. The neutralization of haemolytic haloes was carried out by mixing a constant amount of venom with different amounts of TISCE, and incubated for 30 minutes (min) at 37°C and then, aliquots of the mixtures added to wells of agarose plate. The control well applied with venom solution only. Plates were incubated at 37°C for 20 hrs, and the diameter of haemolytic haloes was measured using vernier calliper (Gutiérrez et al. 1988,Biondo et al. 2003).

Neutralization of venom haemorrhagic activity (*In vivo*)

The venom MHD was determined by injecting 300 µl different concentrations of ICV, CKV, RVV, and SCV intradermally to swiss albino mice. After 3 hrs mice were euthanized using an approved procedure, the area of injected skin is cut, and the haemorrhagic lesion measured, the venom dose that gives the lesion of 10 mm was selected as MHD of particular venom. Challenge dose two MHD was mixed with various concentrations of TISCE and incubated for 30 min at 37°C, aliquots of 300 µl of the mixture were injected intradermally to mice, and observed the results. For control two MHD challenge dose of venom was injected into a control group of mice (R. D.G. Theakston and Reid 1983, Wachtel and Cole 1964).

Neutralization of venom procoagulant effect

Determination of minimum coagulant dose performed as described by Theakston and Reid (1983). The minimum coagulant dose plasma (MCD-P) is the concentration of venom that clots standard citrated human plasma in 60 seconds (sec) at 37°C. In neutralization assays, three MCD-P of venom was mixed with various concentrations of TISCE. The mixtures incubated for 30 min at 37°C. For control, plasma treated with venom alone. The formation or absence of clots is observed during a maximum period of 30 min (Alam and Gomes 2003,R.D.G. Theakston and Reid 1983,Meenatchisundaram et al. 2009)

Proteolytic activity

Proteolytic activity was performed by the method described by Okoromaet al.,(2012). The ICV, CKV, RVV, and SCV were loaded into 3 mm diameter wells of casein-agarose plates (0.1 M Tris-HCl buffer, pH 8.0), and incubated for 24 hrs at 37°C. Then the concentration of venoms was selected which shows the zone diameter of 11 mm. For the neutralization study, the challenge dose of venom was pre-incubated with various concentrations of TISCE for 1 hour (hr) at 37°C and loaded to 3 mm diameter wells of casein-agarose plates, incubated for 24 hrs at 37°C. Plates stained with Coomassie Brilliant Blue-G 250 for 5 min, and destained with methanol: water: acetic acid (50:40:10 v/v). The percentage of neutralization was calculated by measuring the zone of clearance (Okoroma et al. 2012,Gopi, Renu, and Sannanaik 2015).

Evaluation of antivenom activity of TISCE (*In vivo*)

A challenge dose of venom (three LD_{50}) was mixed with various concentrations of the TISCE, and adjusted to a final volume 0.5 ml with normal saline, incubated for 30 min at 37°C, and then aliquots of a final volume of 0.5 ml of each venom mixture injected into groups of six mice. A control group of mice injected with a venom challenge dose alone (Biondo et al. 2003).

Simulation study

In the simulation study, a challenge dose of venom (three LD_{50}), and various concentrations of the TISCE ranging from 50 µg – 300µg was prepared in normal saline. The intravenous route of administration used to inject venom dose into mice and after 5 min interval TISCE injected. A control group injected with a venom challenge dose alone.

Statistical analysis

Experiments were performed with proper controls, and in triplicates. The values are expressed as mean ±S.D. The data were plotted using the GraphPad Prism 8.4.0 software, and the analysis was performed with simple linear regression. Results were considered statistically significant at $p<0.0001$.

Results:-

Preliminary phytochemical analysis of TISCE

Preliminary phytochemical analysis of TISCE was performed to analyze chemical constituents present in TISCE.

Table 1:- Preliminary phytochemical analysis of TISCE.

Sr. no.	Chemical constituents	Test	Observation
1	Detection of carbohydrates	1.Molisch's test 2. Fehling's test	Present
2	Detection of alkaloids	1.Mayer's test 2. Wagner's test	Present
3	Detection of glycoside (cardiac)	1.Keller-killiani test	Absent
4	Detection of saponins	Froth test	Present
5	Detection of phenolic compounds and tannins	1. Ferric chloride test 2. Acetic acid solution 3. Lead acetate solution	Present

Toxicity study of TISCE

This study shows concentrations of TISCE ranging from 100 µg - 300 µg / 0.5 ml were prepared in normal saline solution, and filtered. TISCE is found to be safe, and doesn't show any toxicity signs like excitability, dullness, and weight gain up to 7 days.

Venom characterization

Table 2:- Venom characterization: LD_{50} of venom and its protein content.

Sr. no.	Name	LD_{50} of venom	Protein content (%w/w)
1	ICV	10.0 µg	0.9530
2	CKV	2.4 µg	0.9589
3	RVV	7.4 µg	0.9246
4	SCV	14.0 µg	0.8867

Neutralization of venom haemorrhagic activity (*In vitro*)

To evaluate neutralization of TISCE MHD of the venoms were determined and observed that MHD of ICV (0.2 µg), CKV (0.1 µg), RVV (0.2 µg), and SCV (0.4 µg). TISCE shows the complete neutralization of five MHD of all venoms, TISCE required for complete neutralization of CKV is 5.0 µg, which is least amongst the venoms, ICV, RVV, and SCV required 12.5 µg, 10.0 µg, and 12.5 µg of TISCE respectively (Fig. 1).

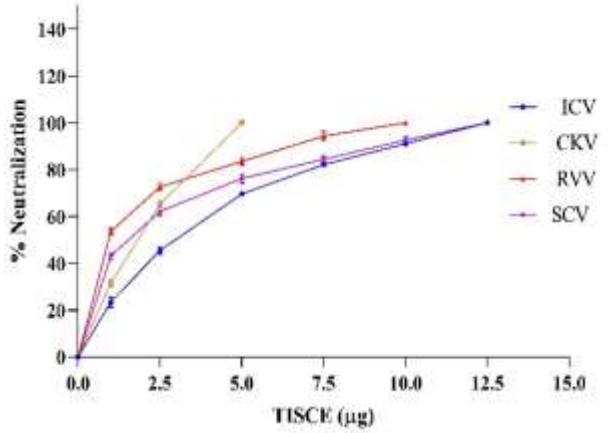

Fig.1:- Neutralization of MHD (*In vitro*). Venoms were pre-incubated with TISCE (0-12.5µg) at 37°C. Data expressed as mean ± S.D. of three independent experiments, p<0.0001.

Neutralization of venom hemorrhagic activity (In vivo)

The hemorrhagic dose of the RVV, and SCV venom producing hemorrhoid lesion of 10 mm diameter at a concentration of RVV at 3 µg, and SCV at 3 µg to injected intradermally under the skin. Challenge dose is selected as two MHD, and neutralization by TISCE is studied. Complete neutralization of challenge dose of RVV is found at 25 µg, and SCV at 30 µg of TISCE (Fig.2). The ICV and CKV not showed any hemorrhoid lesion at concentration 1 µg - 4 µg.

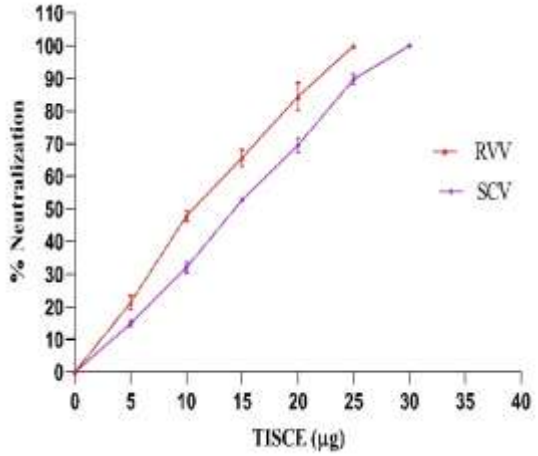

Fig. 2:-Neutralization of MHD (*In vivo*).RVV and SCV were pre-incubated with TISCE (0 - 30 µg) at 37°C. Data expressed as mean ± S.D. of three independent experiments, p <0.0001.

Neutralization of venom procoagulant effect

The MCD-P dose-finding study was performed for ICV, CKV, RVV, and SCV, and only SCV showed a coagulation effect on citrated plasma. SCV coagulated plasma at a concentration of 2.0 µg within 60 sec. To observe the procoagulant effect of the TISCE challenge dose of three MCD-P of SCV and different concentrations of TISCE preincubated mixture was added to plasma and observed for 30 min, it was observed that TISCE (18 µg) can prolong coagulation of plasma up to 30 min (Fig.3).

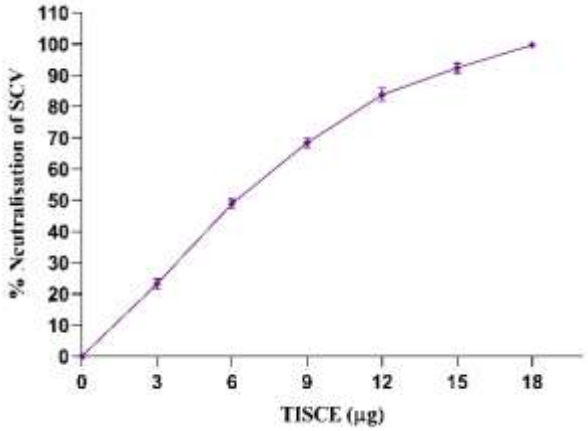

Fig.3:- Neutralization of venom procoagulant effect. SCV was pre-incubated with TISCE (0 -18 µg) at 37°C. Data expressed as mean ± S.D. of three independent experiments, $p<0.0001$.

Neutralization of proteolytic activity

As per the method described by Okoroma proteolytic activity plate assay performed for ICV, CKV, RVV, and SCV, challenge dose is 50 µg, and their neutralization by TISCE is found dose-dependent (Fig.4).

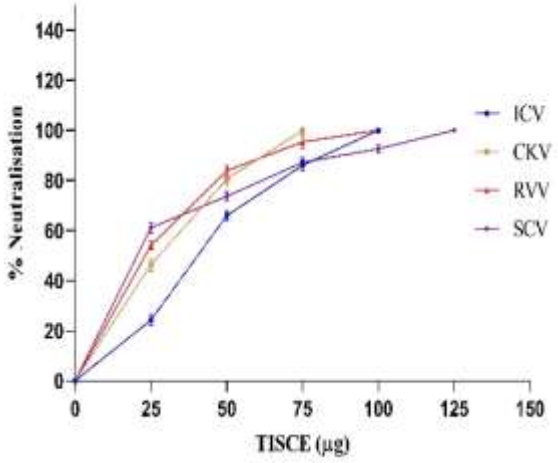

Fig.4:- Neutralization of proteolytic activity by TISCE. Venoms were pre-incubated with TISCE (0 – 125µg) at 37°C. Data expressed as mean ± S.D. of three independent experiments, $p<0.0001$.

Evaluation of venom neutralization activity of TISCE (In vivo):

The three LD_{50} venom challenge dose given to the mice with the different concentrations of TISCE, and calculations performed by the Reed-Muench method. 1.00 mg of TISCE neutralizes 2.23 mg of ICV, 0.536 mg of CKV, 1.65 mg of RVV, and 2.55 mg of SCV.

Discussion:-

In India, herbal plants have been widely used as folk medicine in the treatment of snakebite. The present study provides experimental evidence of TISCE as an antidote for ICV, CKV, RVV, and SCV envenomations. The snakes selected for the study are medically important species of India, which are responsible for a large number of

snakebites and mortalities. The seed coat of the TI seed showed higher activity than whole seed extract, as it is rich in alkaloids, carbohydrates, polyphenolic compounds, saponin glycosides, tannins, and whereas the kernel contains starch as a major component with minimal anti-venom activities compared to TISCE therefore TISCE was continued for further studies. The study revealed that a dose of TISCE up to 300 µg does not show any toxic effects to the mice.

Heamotoxic venoms like RVV and SCV contents Zinc-dependent metalloproteases, which preliminary responsible for the local haemorrhagic effects (R D G Theakston and Reid 1983). The *in-vitro* MHD study results reported that CKV venom showed the highest haemorrhagic halo, when compared to the *in-vivo* study SCV highest haemorrhagic halo indicating the importance of *in-vivo* study during the snake venom research.

Snake venom contains many proteins and enzymes, prothrombin, factor X, and factor V are responsible for actuation of proteolytic enzymes present in the hemotoxic venom. While performing MCD-P, it was observed that RVV not showed any coagulation of plasma, because PLA2 enzymes inhibit blood coagulation by the physical destruction of a factor that contributes directly to the coagulation, also RVV venom does not contain clotting factor V activator. The ICV, and CKV shows the absence of prothrombin factor X activator so not able to coagulate the plasma (Kini 2006, R. D.G. Theakston and Reid 1983). SCV showing procoagulant activity, which was completely neutralized by TISCE.

Proteases catalyze proteolysis of the substrate resulting in the breakdown of the substrate into smaller polypeptides. It was reported that phenolic compounds form hydrogen bonds, and bind strongly with histidine residue present in zinc-binding motifs of metalloprotease, resulting in a decrease of hydrolytic activity (Gopi, Renu, and Jayaraman 2014, Pithayanukul 2009). TISCE shows the complete neutralization of the proteolytic activity of ICV, CKV, RVV, and SCV.

Snake venom has numerous neurotoxic, myotoxic, haemorrhagic, and coagulant compounds that react systemically on the victim's body resulting in the death. In our study, we observed that a pre-incubated mixture of venom and TISCE when injected to the mice shows the complete survival of mice indicating complete neutralization of venom indicating the efficacy of TISCE.

However, during simulation study TISCE able to prolong the deaths of mice but not able to survive them, indicating TISCE may react with venom within certain proximity.

Conclusion:-

In this study, we reported TISCE was exhibited excellent antivenom activities against India's big four snake venom. The TISCE able to prolong the deaths of mice during simulation study, therefore it may be used in the topical applications that will reduce the local venom reactions in the victim, which collectively with ASV may be used to treat envenomed patients more effectively.

However, further studies will require exploring the principal components of TISCE involved in the neutralization with its mode action on snake venom component.

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Declaration of interest

There is no conflict of interest.

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