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Phytochemical Screening, Extraction and In-vivo study of Immunomodulation effect of *Withania somnifera, Momordicadioica* and *Annonasqumosa* leaves

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Abstract:
Phytochemicals and natural products are plant derived secondary metabolites, which may exert many biological activities in humans, including anticancer properties. Certain natural and synthetic compounds that can modulate immune responses in a positive or negative manner are known as Immunomodulators. The best types of nanoparticles used for drug delivery and the biodistribution of phytochemicals and natural products include polymer nanoparticles, solid lipid nanoparticles (SLNs), crystal nanoparticles, liposomes, micelles, and dendrimers We tried various solvent for extractions i.e. Petroleum ether (40-60°c), chloroform, ethanol, water but we get better yield in ethanol. The extracts obtained were then subjected to qualitative chemical examination for the identification of various plant constituents. The various tests and reagents used i.e. Test for Carbohydrates, Test for Proteins, Test for Fats and Oils, Test for Steroids, Test for Glycosides, Test for Alkaloids, Test for Flavonoids, Tests for Tannins and Phenolic Compounds. The ethanol extract of *Annona Squmosa* (ASA), *Momordica Dioica* (MDA) and *Withania Somnifera* (WSA) showed the presence of major phytoconstituents and used as successive ethanol extract (SEE) for in-vivo studies. The results of the acute toxicity study indicated that the LD50 of the extracts was more than 2000 mg/Kg body weight. The in-vivo study results indicate that the extract has a greater effect on the early hypersensitivity reaction and a less pronounced effect on the delayed hypersensitivity reaction.

Keywords: Immunomodulation effect**,** *Withaniasomnifera*, *Momordicadioica* and *Annonasqumosa* leaves

Introduction:

Natural products and compounds have been used as herbal medicines from the beginning of human history. Phytochemicals and natural products are plant derived secondary metabolites, which may exert many biological activities in humans, including anticancer properties. Certain

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natural and synthetic compounds that can modulate immune responses in a positive or negative manner are known as Immunomodulators. Plants have been used for the prevention and cure of various ailments including microbial and lifestyle diseases. According to the World Health Organization (WHO), approximately three quarter of the world‘s population relies on herbal medicine. The implementation of nanotechnology processes involving the improvement of the pharmacokinetics and pharmacodynamics of phytochemicals and natural compounds has gained the focus of researchers, who have developed several innovative delivery systems, including liposomes and polymeric nanoparticles. The biggest issues related to the use of natural products in the treatment of cancer and other diseases are their low solubility and bioavailability, which has caused problems in clinical trials. In this regard, nanotechnology and nanocarrier design may address this issue to improve drug delivery, biodistribution, biosolubility, and bioavailability of natural products and phytochemicals in order to extend the use of these substances in clinical practice.In recent years, studies have allowed the discovery of the specific molecular mechanisms responsible for the therapeutic effects of traditionally used phytochemicals and natural compounds, as well as potentiating their use with nanomedicine. The flexible surface chemistry of nano drug delivery systems also allows the ability to conjugate targeting ligands. Biological moieties such as peptides, nucleic acids, and antibodies can be attached to their surfaces to target drugs to specific diseased sites. Targeted nano drug delivery system (nano-DDS) can increase the drug payload while significantly reducing various risks of adverse side[1-5].

The best types of nanoparticles used for drug delivery and the biodistribution of phytochemicals and natural products include polymer nanoparticles, solid lipid nanoparticles (SLNs), crystal nanoparticles, liposomes, micelles, and dendrimers. Each of these nanoparticles has its own advantages and disadvantages as a drug delivery vehicle. Plant extracts and phytocompounds are found to fortify the host‘s immune system, and numerous plants have been listed in this category. Phytoimmunomodulatory agents can increase the body‘s immune-responsiveness against pathogens by activating the immune system in a specific or a non-specific manner that includes both the innate and adaptive immune systems. The use of plants for immunomodulation can be traced back to ancient Ayurveda of 6000 BC. This system describes medicinal plants as rasayanas having rejuvenating properties in terms of fortifying the immune system against various diseases. Currently, 34 plants have been identified as immunomodulators in rasayana. A number of medicinal plants are currently under high throughput screening for a quick assessment of pharmacologically important hit molecules that could be utilized as a lead molecule in drug development[6-10].

Collection and authentication of plant *Annonasqumosa, Momordicadioica* and *Withaniasomnifera*

This plants are widely distributed in the region of Marathwada and rest of Maharashtra in the hilly and forest area. Generally fruiting in the month from June to December. The plants were collected from Latur Maharashtra. Further its taxonomic identification and authentication was done by Botanist[11-13].

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Standardization of *Annonasqumosa, Momordicadioica* and *Withaniasomnifera leaves (Chengyao et al, 2017; Reddy et al, 2015):*

I. ***Annonasqumosa* leaves**
   a. **Botanical name:** *Annonasqumosa* Linn.
   b. **Synonym:** Sitaphal
   c. **Family:** Annonaceae
   d. **Plant part selected for study:** Leaves

II. ***Withaniasomnifera* leaves**
   a. **Botanical name:** *Withaniasomnifera* (L.) Dunal
   b. **Synonym:** Ashwagandha, winter cherry
   c. **Family:** Solanaceae
   d. **Plant part selected for study:** Leaves

III. ***Momordicadioica leaves***
   a. **Botanical name:** *Momordicadioica*
   b. **Synonym:** Spiny gourd, spine gourd
   c. **Family:** Cucurbitaceae
   d. **Plant part selected for study:** Leaves

Microscopic and Powder Characteristics

1. **Preliminary Pharmacognistic Characteristics**
   In the present study, the *Annonasqumosa*, *Momordicadioica* and *Withaniasomnifera* leaves were investigated for its Macroscopic and Microscopic Characteristics.
2. **Powder Characteristics**
   In the present study, the *Annonasqumosa*, *Momordicadioica* and *Withaniasomnifera* leaves were pulverized into fine powder separately. The powder was investigated for their powder microscopic characteristics.

   The results of the Morphologic and Microscopic characteristics of *Annonasqumosa*, *Momordicadioica* and *Withaniasomnifera* leaves are given in table.

Physical evaluation of *Annonasqumosa, Momordicadioica* and *Withaniasomnifera*leaves

The authenticated shade dried leaves of plant namely *Annonasqumosa*, *Momordicadioica* and *Withaniasomnifera* were subjected to size reduction to get the coarse powder of drug and then passed through sieve no. 45 to get the uniform powder.

Following parameters enlisted were done; Extractive values, Ash values, Moisture content (Loss on Drying)

EXTRACT PREPARATION OF *ANNONA SQUMOSA, MOMORDICA DIOICA* AND *WITHANIA SOMNIFERA* LEAVES

25g of the sample was weighed accurately shade drying of leaves and homogenization to coarse powder, Soxhlet extraction (successive solvent extraction) using following solvents Petroleum ether (40-60°c), chloroform, ethanol, water. Concentration of extracts using water bath and dried. Its yield which is illustrated in table.

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Phytochemical Screening:

The extracts obtained were then subjected to qualitative chemical examination for the identification of various plant constituents. The various tests and reagents used are given below: Test for Carbohydrates, Test for Proteins, Test for Fats and Oils, Test for Steroids, Test for Glycosides, Test for Alkaloids, Test for Flavonoids, Tests for Tannins and Phenolic Compounds.

The results of the qualitative chemical test of *Annona Squmosa*, *Momordica Dioica* and *Withania Somnifera* leaves are given in the table.

In-Vivo Study

Materials & Methods:

Preparation of extracts

The powder was subjected to Soxhlet extraction using different solvents of varying polarity. The extracts were subjected to qualitative chemical tests to determine the nature of the phytoconstituents.

An aqueous dispersion of the successive ethanol extract (SEE) was used for in-vivo animal experiments. The vehicle (distilled water) served as the control.

Animals

Random albino rats of both sexes were used for acute toxicity and pharmacological studies. The animals were maintained at room temperature and fed with standard pellet diet and tap water.

Antigen

Sheep red blood cells (SRBCs), collected in Alsevier's solution, washed in large volumes of sterile normal saline thrice and adjusted to a concentration of 5×10^9 cells per ml, were used for immunisation and challenge.

Reagents

The minimum essential medium (MEM) used for bioassay was procured from HiMedia Lab Pvt. Ltd. Ficoll Hypaque and bovine serum albumin were procured from Sigma Chemical Co. All the solvents, reagents and chemicals used were of analytical grade.

***In-vivo* tests**

Acute toxicity study:

The acute toxicity study for the extracts was conducted in rats as per the prescribed guidelines. Three animals of either sex were used. The weights were recorded before beginning the study. They were administered a single bolus dose of the extract (2000 mg/kg) per orally and observed over 14 days for mortality and physical/ behavioural changes.

Hypersensitivity reaction which measures cellular immunity:

Hypersensitivity reaction to SRBC was induced in rats, following the prescribed method. The extracts (in doses of 50, 100 and 200 mg/kg, body weight) was administered to the animals (test group) orally for five days and the vehicle was administered to the control animals. Each group consisted of six rats-three male and three female. The extracts were administered orally on each of the two days prior to the immunization, on the day of the immunization and on each of the two days after the immunization (i.e., Days -2,-1, 0,+1,+2).

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The rats were immunized by injecting 0.1 ml of SRBC subcutaneously into the right hind footpad on day 0. The animals were challenged seven days later by injecting the same amount of SRBC into the left hind footpad. The thickness of the left hind footpad was measured with a micrometer at 4 h and 24 h after the challenge.

Hemagglutination reaction which measures the humoral immunity

The extracts (in doses of 50, 100 and 200 mg/kg, body weight) was administered to the animals (test group) orally for five days and the vehicle was administered to the control animals. Each group consisted of six rats- three male and three female. The extracts administered orally on each of the two days prior to the immunization, on the day of the immunization and on each of the two days after the immunization (i.e., Days -2, -1, 0, +1, +2).

The rats were immunized by injecting 0.5 ml of SRBCs intraperitoneally (i.p.) on the day of the immunization. Blood samples were collected by retro-orbital puncture on the tenth day after the immunization. Antibody levels were determined by the hem agglutination technique. The antibody titer was determined by a two-fold serial dilution of one volume (100 µl) of serum and one volume (100 µl) of 0.1 % bovine serum albumin (BSA) in saline. One volume (100 µl) of 0.1% SRBCs in BSA in saline was added and the tubes were mixed thoroughly. They were allowed to settle at room temperature for about 60-90 min until the control tube showed a negative pattern (a small button formation). The value of the highest serum dilution showing visible hem agglutination was taken as the antibody titer.

Results:

Standardization of Bark

Table 1: Physico-chemical parameters of *Annona Squmosa* (ASA), *Momordica Dioica* (MDA) and *Withania Somnifera*(WSA)

Sr. No.	Standardization Parameter	*Annona Squmosa*(ASA)	*Momordica Dioica* (MDA)	*Withania Somnifera* (WSA)
1	% Foreign Organic Matter (w/w)	< 2	< 2	< 2
2	% Total Ash (w/w)	7.52	7.46	7.41
3	% Acid Insoluble Ash (w/w)	0.31	0.33	0.36
4	%Water Soluble Ash (w/w)	1.57	1.62	1.51
5	Sulphated Ash Value (%)	0.946	0.952	0.934
6	Moisture Content (w/w)	1.462	1.469	1.458
7	% Extractive Values (w/w)	1.15	1.13	1.14
8	Alcohol Soluble	25.81%	25.42%	25.57%
9	Water Soluble	43.49%	43.34%	43.63%

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Table 2: Phyto-chemical investigation of *Annona Squmosa* (ASA), *Momordica Dioica* (MDA) and *Withania Somnifera* (WSA)

Test	Pet. Ether extract			Chloroform extract			Ethanol extract			Aqueous extract		
	ASA	MDA	WSA	ASA	MDA	WSA	ASA	MDA	WSA	ASA	MDA	WSA
Glycosides	-	+	+	-	+	+	+	+	+	+	+	+
Phytosterol	+	+	-	-	+	-	-	+	-	-	+	-
Alkaloids	-	+	+	+	+	+	+	+	+	+	+	+
Oils	+	+	+	-	+	+	-	+	+	+	+	+
Saponins	-	+	+	-	+	+	-	+	+	-	+	+
Phenols	-	+	+	-	+	+	+	+	+	+	+	+
Flavonoids	-	+	+	-	+	+	+	+	+	+	+	+

[Note: +sign indicate Presence whereas –sign indicate Absence]

Acute Toxicity Study:

The results of the acute toxicity study indicated that the LD_{50} of the extracts was more than 2000 mg/Kg body weight.

Hypersensitivity reaction:

Per oral administration of the extracts (50, 100 and 200 mg/kg) for five days produced a dose related increase in early (4h) and delayed (24h) hypersensitivity reaction in rats. The 4 hour-reaction was found to be of higher magnitude than the 24 hour-reaction.

Hem agglutination reaction:

The antigen antibody reaction results in agglutination. The relative strength of an antibody titer is defined as the reciprocal of the highest dilution which is still capable of causing visible agglutination. The antibody titer is useful to measure the changes in the amount of the antibody in the course of an immune response.

Per oral administration of the extracts (50, 100 and 200 mg/kg) for five days produced a dose related increase in the antibody titer in rats.

Table 3: Hypersensitivity and hemagglutination reactions of the successive ethanol extract

Extract (mg/kg, p.o.)		Hypersensitivity reaction		Hem agglutination antibody titer	
		4 h (mean±SD)	24 h (mean±SD)	Range	mean±SD
Control		0.54±0.10	-0.02±0.32	128-256	224±71.3
50		0.72±0.19	0.26±0.31	128-1024	445±318.6
100		0.85±0.11	0.49±0.23	256-2048	1051±548.6
200		0.97±0.21	0.63±0.22	512-2048	1134±621.8
One-way ANOVA	F	5.46	5.84		7.21
	P	<.05	<0.05		<0.05

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Values are mean ±SD, n=6 in each group. df=3, 20, *P<.05 when compared with control group (Dunnett's test). Inflammation after challenge. The differences in rat paw thickness before and after the antigen in mm.

Conclusion:

The ethanol extract of *Annona Squmosa* (ASA), *Momordica Dioica* (MDA) and *Withania Somnifera* (WSA) showed the presence of major phytoconstituents and used as successive ethanol extract (SEE) for in-vivo studies. The results of the acute toxicity study indicated that the LD_{50} of the extracts was more than 2000 mg/Kg body weight. The in-vivo study results indicate that the extract has a greater effect on the early hypersensitivity reaction and a less pronounced effect on the delayed hypersensitivity reaction.

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