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EXPERIMENTAL EVALUATION OF CELL-MEDIATED IMMUNITY OF HERBO-MINERAL FORMULATION R9 VATI

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Abstract

Introduction: The concept of well-being has evolved to encompass not just the absence of illness but also overall health. Rasayana, referring to the nutritional essence that penetrates all body tissues to replenish and nurture them, plays a key role. It enhances longevity, intelligence, youthful vigor, skin health, voice quality, and the robustness of both motor and sensory functions. This is achieved through the use of rasayana herbs, typically administered in powdered or decoction forms, which fortify various dhatu (tissues) and promote overall well-being.

The herbs like ashwagandha, pippali, guduchi, and yashtimadhu, all possessing potent antioxidant properties. Rasasindura, a preparation containing purified mercury (shuddha parada) and sulfur (shuddha gandhaka), is a meticulously processed formulation utilized as a therapeutic agent in treating various ailments. When incorporated into formulations, these Rasaushadhi (herbal medicines processed with metals and minerals) augment the efficacy of herbal remedies, often requiring only small amounts to yield significant effects.

To explore the additional benefits of incorporating Rasasindura into a blend of herbal medicines known for their antioxidant properties, an experimental study was conducted on the test formulation.

Method: Evaluation of cell-mediated immunity, Assessment of humoral immunity through antibody production, Antibody response against SRBC.

Observation and result: R9 V and R9 VWS showed non-significant stimulation of the anti-body formation. The antibody formation represents expression of Humoral immunity hence based on the results obtained it can be inferred that test drug both have mild to moderate Immuno stimulant effect.

Key words Rasayana, R9 Vati, Immune Modulator, experimental study, Rasasindura, anti-oxidant

Introduction

Any organism can fall prey to disease-causing agents, even at a microscopic scale where a million bacteria can inhabit the head of a pin. Despite this, bacteria possess defense mechanisms against viral invaders, a defense that strengthens with organism complexity. In addition to antimicrobial treatments, current approaches to illness should aim at bolstering an individual's immunity. This strategy, involving the use of herbs and medications known as rasayana, replenishes vital nutrients and enhances vitality[1]. With the modern understanding of health extending beyond mere absence of illness, the significance of well-being has grown. This underpins the concept of functional foods, even within traditional frameworks, utilizing herbs to both treat ailments and enhance quality of life-an ancient practice known as rasayana treatment.

While traditional herbal preparations like powders and decoctions remain relevant, modern culture demands a more systematic approach to their usage due to perceived complexities and time constraints. Science has investigated and validated the effects of many Rasayana plants on the immune system, endocrinological benefits, antioxidant properties, memory and learning behavior enhancement, etc. Despite the fact that the concept is well-known and frequently applied, it is required to rationalize the approach and investigate the possible phyto-chemical components that could account for the majority of these herbs actions and activities[2].

The Ayurvedic Rasayana idea may be the best example of complementary therapies with health benefits. Rasayana means "path or direction of the elixir of life" because it literally translates to Rasa (elixir) and Ayana (home or road). The Ayurvedic approach to disease prevention and health promotion is becoming more and more popular in the modern world[3]. One of the eight primary subdisciplines of Ayurveda is rasayana therapy. The term "Rasayana" describes the path that the nutritive essence (Rasa-dhatu) takes to go to every part of the body so that it can replace and nourish it. The father of Ayurveda, Charaka, describes rasayana as a way to combine the greatest qualities of different dhatu (tissues). Rasayana therapy improves skin, voice, motor skills, youth, lifespan, and intelligence.

The anti-oxidant qualities of ashwagandha, pippali, guduchi, and yashtimadhu - also known as rasayana in Ayurvedic literature - have been shown in numerous research. A rasashaudhi called rasasindura, also referred to as rasayana, is recommended for a variety of ailments. When used with the previously stated herbs, this may have a synergistic effect on the composition. The current formulation's bhavana was made up of shunti, tulasi, maricha, and twak, which are also known as rasayana in Ayurvedic literature. They also have immunomodulatory and antioxidant properties, according to earlier research. It is also recognized that the bhavana process increases the drug's potency. These were therefore picked for the bhavana. The formulation was chosen for the investigation because it will be more economical and effective.

Many forms of inorganic mercury compounds, principally mercuric sulphide (HgS), have been employed in Indian and Chinese traditional medicine from ancient times[4]. Among the several Hg-based medicines that are currently widely used in Ayurveda to treat a range of chronic ailments, such as syphilis, pleural effusion, high fever, and mental problems, are rasasindura, kajjali, and makardhwaja[5]. The immuno-modulatory role of these Hg-based Ayurvedic drugs accounts for their success. These drugs are believed to be antioxidants and are used as a rejuvenating agent[6].

Ayurvedic medicine, despite containing Hg, has not shown any harmful effects in recent in-vitro and in-vivo studies on Hg-based therapies. In contrast to Chinese medicine, where cinnabar ore is used directly, Ayurvedic kajjali and rasasindura treatments involve a meticulous process of preparing mercury and sulphur, including cleaning, combining, and heat treatment. During the creation of rasasindura, kajjali transforms into the crystalline form of β -HgS, which is utilized as a medicinal substance. The final product, rasasindura, consists of a single crystalline α -HgS phase that is highly pure, containing minimal impurities[7].

Kajjali plays a crucial role in the preparation of rasasindura and exists in the form of β-HgS crystals. It is also utilized as a medicinal substance. The final product, Rasasindura, is a highly pure single crystalline α-HgS phase with a small amount of impurity. These Ayurvedic medicines contain nano-sized particles (100nm) with a stable crystal structure and minimal crystal defects (less than 3%). Hence, it can be inferred that the unique Ayurvedic preparation techniques are responsible for the efficacy of these Hg-based medications[8].

Antioxidants play a crucial role in safeguarding our health and are vital for human survival. Moreover, they are widely used as additives in fats and oils. In the food processing industry, antioxidants are utilized to prevent or delay food spoilage. Medicinal plants have gained immense popularity as sources of antioxidants for treating various ailments. Antioxidants are hailed as the driving force behind human progress and existence due to their ability to combat free radicals, metabolic disorders, and age-related syndromes in both humans and animals. In this specific study, the immune-modulating effect of R9 Vati was examined through experimental investigation, both with and without the addition of rasasindura.

Objective: Assessment of cell mediated immunity

Materials and methods

Animal grouping

The selected animals were grouped into different groups randomly irrespective of sexes and each group comprised of six animals.

GROUP 1 - water control + triple antigen sensitization

GROUP 2 - Vehicle control (1% CMC)+ triple antigen sensitization

GROUP 3 - R9 V+ triple antigen sensitization

GROUP 4 - R9 VWS+ triple antigen sensitization

The rats were sensitized subcutaneously (1 ml /100 g body weight) on first day of drug administration with the following solution;

Triple antigen (TPA) = 1 ml

Normal saline (NS) = 4 ml

Potash alum (10%) = 1 ml

PH of the above reagent (that is potash alum adjuvant) will be maintained between 5.6 – 6.8 using 10 % sodium carbonate

Table 1: Showing body weight and dose of TPA injected in normal control group (water control + triple antigen sensitization)

ANIMAL	BODY WEIGHT	TPA injected(1ml/100g) in dorsal area of rat
HEAD	185 g	1.85 ml
NECK	168 g	1.68 ml

BODY	180 g	1.80 ml
TAIL	170 g	1.70ml
FORELIMB	175 g	1.75ml
NO MARK	185 g	1.85ml

Table 2: Showing body weight and dose of TPA injected in vehicle control group (vehicle control + triple antigen sensitization)

ANIMAL	BODY WEIGHT	TPAinjected(1ml/100g) in dorsal area of rat
HEAD	200 g	2.00 ml
NECK	174 g	1.74 ml
BODY	193 g	1.93 ml
TAIL	180 g	1.80 ml
FORELIMB	164 g	1.64 ml
NO MARK	172 g	1.72 ml

Table 3: Showing body weight and dose of 1% CMC injected in vehicle control group from 3rd day to 7th day:

ANIMAL	BODY WEIGHT	1% CMC (1ml/100 g)
HEAD	200 g	2.00 ml
NECK	174 g	1.74 ml
BODY	193 g	1.93 ml
TAIL	180 g	1.80 ml
FORELIMB	164 g	1.64 ml
NO MARK	172 g	1.72 ml

Table 4: Showing body weight and dose of TPA injected in test group I (R9 V+ triple antigen sensitization)

ANIMAL	BODY WEIGHT	TPA injected (1ml/100g) in dorsal area of rat
HEAD	187 g	1.87 ml
NECK	158g	1.58 ml
BODY	204g	2.04 ml
TAIL	210 g	2.10ml
FORELIMB	149 g	1.49 ml
NO MARK	180g	1.80 ml

Table 5: Showing body weight and dose of Test group I(R9 V+ triple antigen sensitization)

ANIMAL	BODY WEIGHT	Dose of R9 V (200mg/kg)
HEAD	187 g	1.87 ml
NECK	158g	1.58 ml

BODY	204g	2.04 ml
TAIL	210 g	2.10 ml
FORELIMB	149 g	1.49 ml
NO MARK	180g	1.80 ml

Solution -400 mg (R9 V) +20ml (distilled water)

Dose of R9 V-200mg/kg

Table 6: Showing body weight and dose of TPA injected in test group II (R9 VWS+ triple antigen sensitization)

ANIMAL	BODY WEIGHT	TPA injected(1ml/100g) in dorsal area of rat
HEAD	175 g	1.75 ml
NECK	183 g	1.83 ml
BODY	192 g	1.92 ml
TAIL	248 g	2.48 ml
FORELIMB	195 g	1.95 ml
NO MARK	192 g	1.92 ml

Table 7: Showing body weight and dose of Test group II (R9 VWS+ triple antigen sensitization)

ANIMAL	BODY WEIGHT	Dose of R9 VWS (200mg/kg)
HEAD	175 g	1.75 ml
NECK	183 g	1.83 ml
BODY	192 g	1.92 ml
TAIL	248 g	2.48 ml
FORELIMB	195 g	1.95 ml
NO MARK	192 g	1.92 ml

Solution -400 mg (R9 VWS) +20ml (distilled water)

Dose of R9 VWS-200mg/kg

On seventh day the initial paw volume of left hind paw were noted and 0.1 ml of TPA solution were injected into plantar aponeurosis of left hind paw, volume of immunological edema thus produced was measured by volume displacement method, after 24 hours and 48 hours with plethysymograph . Percentage increase in paw volume, which is the induced edema formation over initial value, was calculated. The values from control group were compared with the values from test drug administered groups to assess the cell mediated immunity response of the drug

Assessment Of Immunity By Humoral Anti-Body Formation

Materials and methods:

1. Wister strain albino rats of either sex weighing between 140-280 g either sex was used for experimental study. And 36 albino rats were used for the study.
2. The animals were obtained from the S.D.M central animal house.
3. 2 formulation *(R9 V and R9 VWS)* was subjected for evaluation of immunity and was

studied by Humoral anti- body formation

4. Chemicals used:

All chemicals are analytical grade (EXLR) regularly used in lab.

a) Normal saline
b) 2% Dextrose
c) 0.8% Sodium citrate
d) 0.5% Citric acid
e) 0.42% Sodium chloride
f) 30% SRBC solution (for this purpose the sheep blood would be collected from city slaughter house in a sterilized bottle.)

5. Equipments and glass ware to be used:

Glass tubes, slides, syringes etc.

Route of drug administration

The test drug, vehicle and control would be administered according to the body weight of animals by oral route.

Inclusion criteria

a) Animals will be selected are adult albino rats having weight from 140-280g.
b) Animal will be selected from both the sexes.

Exclusion criteria

a) Unhealthy albino rats.
b) Weight range below 140g and above 280g.

Animal grouping

The selected animals were grouped into different groups randomly irrespective of sexes and each group comprised of six animals.

GROUP 1 - Control group
GROUP 2 - Positive control (1% CMC) + SRBC
GROUP 3 - Test group I (R9 V) + SRBC
GROUP 4 - Test group II (R9 VWS) + SRBC

Assessment criteria

The test drug and vehicle were administered for 10 consecutive days. On third day of test drug administration, the animals were sensitized with 30% SRBC solution. For this purpose the sheep blood was collected from city slaughterhouse in a sterilized bottle containing Alsever`s solution (2% dextrose , 0.8% sodium citrate , 0.5% citric acid ,and 0.42% sodium chloride) aseptically so that agglutination of blood does not take place. The collected sheep blood was thoroughly washed with sterile normal saline through repeated centrifugation until the supernatant fluid become colorless and made to 30% SRBC solution. This sensitizing agent was injected sub-cutaneously in the dose of 1ml/100g of body weight to the rats. On the 10^{th} day the animals were sacrificed by deep ether anesthesia and the blood was collected in sterile test tubes. Serums were separated from it and the components were inactivated by incubating for 30 minutes at 56 ^{0}C temperature in a serological water bath.

The micro-titer plate were filled with 0.1 ml sterile normal saline and serial two fold dilutions of 0.1 ml of the serum in sterile saline solution were made in the micro-

titer plate. 0.1 ml of thrice saline washed 3% SRBC was added to each well of the tray. Blood from the same animal (sheep) was used for both sensitization and to determine antibody titer. The trays were covered and placed in refrigerator overnight. Antibody titer (heamagglutination titer) was noted on the next day. The titers were converted to $\log_2$ values for easy comparison. Spleen and lymph node were dissected out from the sacrificed animals and their weight was recorded. Tissues (including lymph node) were transferred to 10% formaldehyde solution for fixation and later on processed for histological studies.

Table 8: Effect of R9 V&R9 VWS *on* Antibody formation against SRBC (Antibody titer)

Groups	Log 2 value	% change
SRBC Control	3.34 ± 0.11	---
R9 V	3.80± 0.58	13.77 ↑
R9 VWS	4.27 ± 0.92	27.84 ↑

Data: MEAN ± SEM

The data related to the effect of R9V & R9VWS on log 2 values and in terms of percentage change of antibody titration has been shown in table 8. There was a increase in log 2 value of antibody titration of *R9 V* dose and *R9 VWS* when compared to SRBC control group. But the observations were statistically not significant.

Table 9: Effect of R9 V&R9 VWS *on* % change in Body weight:

Groups	% change in body weight	% change
SRBC Control	2.55 ± 0.95	---
R9 V	0.30 ± 1.13	88.23 ↓
R9 VWS	2.97 ± 1.86	16.47 ↑

Data: MEAN ± SEM

The data related to the effect of R9 V&R9 VWS on body weight has been deplicated in table 9

Data showed non-significant decrease in % change in body weight in *R9V* dose when compared to SRBC control group. There was non-significant increase in % change in body weight in R9VWS dose when compared to SRBC control group

Table 10: Effect of R9V & R9VWS *on* weight of Spleen

Group	Spleen weight (G/dl) MEAN ± SEM	% change
Normal control	0.75 ± 0.14	-
SRBC control	0.72 ± 0.08	4 ↓ #
R9 V	0.62 ± 0.06	13.88↓ @
R9 VWS	0.79 ± 0.07	9.72 ↑ @

Data: MEAN ± SEM,

#-compared with normal control, @- compared with SRBC control

The data related to the effect of R9V & R9VWS on spleen weight has been deplicated in table 10. Data showed a decrease in weight of spleen in SRBC control group compared to normal control group. There was decrease in spleen weight of *R9V* dose when compared to SRBC control group. There was an increase in spleen weight of *R9VWS* dose when compared to SRBC control. But observed values are found to be statistically not significant.

Table 11: Effect of R9 V&R9 VWS *on* weight of lymph node

Group	lymph node weight (G/dl) MEAN ± SEM	% change
Normal control	0.30 ± 0.05	-
SRBC control	0.17 ± 0.01	43.33 ↓ #
R9 V	0.28 ± 0.03	64.70↑@
R9 VWS	0.27 ± 0.02	58.82 ↑@

Data: MEAN ± SEM,

#-compared with normal control, @- compared with SRBC control

The data related to the effect of R9 V&R9 VWS on lymph node weight has been deplicated in table 11. Data showed decrease in lymph node weight of in SRBC control group compared to normal control group. There was increase in lymph node weight of *R9 V and R9 VWS* groups when compared to SRBC control group. But data is statistically not significant.

Effect of test drug on immunological paw edema in pre-sensitized albino rats

Table 12: Effect of *TEST DRUG* on DPT induced in paw edema measured after 24 hr challenge

Groups	Paw volume (ml)	% change
Normal Control	68.56 ± 9.49	---
Vehicle control	97.15±10.69*	41.70↑
R9 V	32.92 ±4.87**	51.98↓
R9 VWS	48.38 ± 6.23**	29.43↓

Data: MEAN ± SEM, *P<0.05, **P<0.01

Data related to the effect of TEST DRUG on triple antigen induced paw oedema in rats has been depicted in the Table 12 as actual figures and in terms of percentage increase in the paw volume. Injection of the antigen and adjuvant produced significant paw oedema in the pre-sensitized rats. Data shows there was a very significant increase in paw volume of Vehicle control group when compared to the control group. There was a very significant decrease in paw volume of the test drugs (R9 V and R9 VWS) when compared to the control group.

Table 13: Effect of *TEST DRUG* on DPT induced paw edema measured after 48 hr challenge

Groups	Paw volume (ml)	% change
Control	44.05 ±4.98	---
Vehicle control	75.2 ±9.78*	70.71↑
R9 V	14.73 ± 5.17**	66.56↓
R9 VWS	31.12 ± 7.23	29.35↓

Data: MEAN ± SEM, *P<0.05, **P<0.01

Data related to the effect of TEST DRUGon triple antigen induced paw oedema in rats has been depicted in the Table 13 as actual figures and in terms of percentage increase in the paw volume. Injection of the antigen and adjuvant produced significant paw oedema in the pre-sensitized rats. Data shows there was a significant increase in paw volume of vehicle control group when compared to the control group. There was a very significant decrease in paw volume of the test drugs test drug *(*R9 V) when compared to the control group, there was a non significant decrease in paw volume of the test drugs test drug *(*R9 VWS) when compared to the control group,

Observation and result:

Table 14: Effect of R9 V & R9 VWS *on* Antibody formation against SRBC (Antibody titer)

Groups	**Log 2 value**	**% change**
SRBC Control	3.34 ± 0.11	---
R9 V	3.80± 0.58	13.77 ↑
R9 VWS	4.27 ± 0.92	27.84 ↑

Data: MEAN ± SEM

The data related to the effect of R9 V & R9 VWS on log 2 values and in terms of percentage change of antibody titration has been shown in table 14.

There was a increase in log 2 value of antibody titration of *R9V* dose and *R9VWS* when compared to SRBC control group. But the observations were statistically not significant.

Table 15: Effect of R9 V &R9 VWS *on* % change in Body weight:

Groups	% change in body weight	% change
SRBC Control	2.55 ± 0.95	---
R9 V	0.30 ± 1.13	88.23 ↓
R9 VWS	2.97 ± 1.86	16.47 ↑

Data: MEAN ± SEM

The data related to the effect of R9 V & R9 VWS on body weight has been deplicated in table 15. Data showed non-significant decrease in % change in body weight in *R9V* dose when compared to SRBC control group. There was non-significant increase in % change in body weight in R9VWS dose when compared to SRBC control group

Table 16: Effect of R9 V & R9 VWS *on* weight of Spleen

Group	Spleen weight (G/dl) MEAN ± SEM	% change
Normal control	0.75 ± 0.14	-
SRBC control	0.72 ± 0.08	4 ↓ #
R9 V	0.62 ± 0.06	13.88↓ @
R9 VWS	0.79 ± 0.07	9.72 ↑ @

Data: MEAN ± SEM,

#-compared with normal control, @- compared with SRBC control

The data related to the effect of R9 V & R9 VWS on spleen weight has been deplicated in table 16. Data showed a decrease in weight of spleen in SRBC control group compared to normal control group. There was decrease in spleen weight of *R9 V* dose when compared to SRBC control group. There was an increase in spleen weight of *R9 VWS* dose when compared to SRBC control. But observed values are found to be statistically not significant.

Table 17: Effect of R9 V & R9 VWS *on* weight of lymph node

Group	lymph node weight (G/dl) MEAN ± SEM	% change
Normal control	0.30 ± 0.05	-
SRBC control	0.17 ± 0.01	43.33 ↓ #
R9 V	0.28 ± 0.03	64.70↑@
R9 VWS	0.27 ± 0.02	58.82 ↑@

Data: MEAN ± SEM,

#-compared with normal control, @- compared with SRBC control

The data related to the effect of R9 V & R9 VWS on lymph node weight has been deplicated in table 17. Data showed decrease in lymph node weight of in SRBC control group compared to normal control group. There was increase in lymph node weight of *R9V and R9VWS* groups when compared to SRBC control group. But data is statistically not significant.

Discussion

Consolidated statement of Cell Mediated Immunity:

Table 18: **Consolidated statement of Cell Mediated Immunity**

GROUPS	24th Hour	48th Hour
Vehicle control	SI	SI
R9 V	SD	SD
R9 VWS	SD	NSD

SI - Significantly Increase SD - Significantly Decrease
NSI - Non Significantly Increase NSD - Non Significantly Decrease

Cell mediated immunity is the responsible for delayed type hypersensitivity and certain T cells suppress antibody production. The test sample was evaluated to assess their effect on cell mediated immunity against an experimental model, which is supposed to represent cell mediated immunity.

Analysis of the data show that the vehicle control Group produced weak suppression of immunological edema at 24th and 48th hour after injection of the paw edema eliciting agent. In Test Drug- I (R9 V) administrated group shows remarkable and statistically very significant suppression of paw edema was observed both at 24th and 48th hour post injection. In Test Drug-II (R9 VWS) administrated group shows moderately suppression of paw edema was observed both at 24th and 48th hour post injection. This indicates the effect of the test drug formulation possess very good immunological edema suppression effect. The immunological edemas represents expression of cell mediated immunity hence based on the results obtained it can be inferred that test drug both have CMI suppression effect.

Table 19: **Consolidated statement of effect of R9 V&R9 VWS Anti-body titer**

Parameters	**R9 V**	**R9 VWS**
Antibody titre	**NSI**	**NSI**

Table 20: Consolidated statements

Parameters	**SRBC control#**	**R9 V***	**R9 VWS***
Body weight	--	NSD	NSI
Spleen weight	NSD	NSD	NSI
Lymph node weight	NSD	NSI	NSI

- Compared with normal control *- Compared with SRBC control

Abbreviations used in above table:

NSE - No significant effect; **NSD –** Non-significant decrease
SD – Significant decrease; **NSI –** Non-significant increase

Hem-agglutination antibody titer is a primary parameter for studying the Humoral response. Antigen and antibody reaction results in agglutination. Antibody molecules secreted by plasma cells mediate the Humoral immune response.

The test formulation showed mild increase (stimulation) in of anti-body formation against SRBC sensitized animals where study was done following the model This indicates at the dose level employed *R9 V and R9 VWS* has mild modulatory effect on Th-2 mediated pathway of immune reaction. But the increase in either duration of drug may give significant Immunomodulatory effect.

In present study pre-sensitization to animals with R9 V and R9 VWS administration lead to mild changes in body weight

In present study mild decrease in the weight of spleen and lymph node were observed in SRBC

control group in comparison to normal control group. R9 VWS showed non-significant increase in the weight of the spleen. There was moderate increase in the lymph node weight in both test drug groups. This indicates the stimulation of the activity of these glands. As spleen and lymph nodes were lymphoid organs and play a vital role in immune responses. There will be proliferation of lymphoid tissue in presence of antigen for the production of antibodies hence there will be increase in the weight of organ. This factor is should be supported by Histopathological observation.

In conclusion, R9 V and R9 VWS showed non-significant stimulation of the anti-body formation. The antibody formation represents expression of Humoral immunity hence based on the results obtained it can be inferred that test drug both have mild to moderate Immunostimulant effect.

CONCLUSION

In conclusion, R9 V and R9 VWS showed non-significant stimulation of the anti-body formation. The antibody formation represents expression of Humoral immunity hence based on the results obtained it can be inferred that test drug both have mild to moderate Immuno stimulant effect.

In Test Drug- I (R9 Vati without rasasindura) administrated group shows remarkable and statistically very significant suppression of paw edema was observed both at 24^{th} and 48^{th} hour post injection. In Test Drug- II (R9 with rasasindura) administrated group shows moderately suppression of paw edema was observed both at 24th and 48th hour post injection. Analysis of the experimental study data shows that test drug formulation possesses statically significant immunological edema suppression effect.

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