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PHYTOCHEMICAL ANALYSIS AND IMMUNOMODULATORY ACTIVITY OF ASANA (PTEROCARPUS MARSUPEUM ROXB.) - AN INVITRO STUDY

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ABSTRACT

Introduction: Due to increasing side effects of available modern medicines, Immunomodulation using Ayurvedic drugs specially *Rasayana* drug will be helpful for people in day to day life. *Asana* has been mentioned as *Rasayana* dravya by many Acharyas. Therefore *Asana (Pterocarpus marsupium)* is expected to have immunomodultory activity apart from its other physiological activities. **Aims and objective:** This study is undertaken "To Evaluate Immunomodulatory activity of *Asana* (*Ptreocarpus marsupeum*) in-vitro method. **Materials and methods:** The immunomodulaton activity of Asana (Pterocarpus marsupium) was evaluated using Nitrobluetetrazolium Test (NBT), Neutrophil locomotion & Chemotaxis test, Phagocytosis and candidacidal assay. **Result:** Immunomodulation assay showed that the *Asana* (pterocarpus marsupium) significantly facilitates neutrophil locomotion towards the stimulus or infected site, potentiates neutrophil to kill foreign organism and stimulate of neutrophil for phagocytic activity. From above results of immune-modultory assay with three parameters it is noted that the extract of heart wood of *Asana* (pterocarpus marsupium) shows significant immune-modulatory activity.

KEYWORDS: Asana, Pterocarpus marsupium, Immunomodulation.

INTRODUCTION

The utility of Ayurveda science is to help maintain the health of healthy individual and to cure disease of patient.[1] To fulfill this, Ayurveda has one separate branch known as *Rasayana*. *Rasayana* mainly deals with progress of physical and mental health.

As Acharya Charaka says in Rasayana Adhyaya that, a person undergoing rejuvenation therapy attains Longevity, Memory, Intellect, and Freedom from disease, excellence of Lusture, Complexion and Voice, excellent potentiality of body and organs.[2]

Due to increasing side effects of available modern medicines, Immunomodulation using Ayurvedic drugs specially *Rasayana* drug will be helpful for people in day to day life. The immune system is known to be involved in patho-physiological mechanism of many diseases. Now a day lifestyle and food habits are the reasons of immune system dysfunction due to which the human beings are prone to various major diseases. To prevent this effective Immunomodulatory drugs are needed. Immunomodulatory may be defined as a substance biological or synthetic which can stimulate, suppress or modulate any of the components of immune system.[3] The basic function of our immune system in body is to protect body against infections and pathogenesis. Asana has been mentioned as *Rasayana* dravya by many Acharyas. It has *Kashaya, Tikta rasa,Ushnaveerya, Katuvipaka, Laghu and Rukshaguna* and helps to enhance *Rasayana* effect on body.[3] Therefore *Asana (Pterocarpusmarsupium)* is expected to have Immunomodultory activity apart from its other physiological activities. Hence in this study is undertaken "To Evaluate Immunomodulatory activity of *Asana* (*Ptreocarpusmarsupeum*) in-vitro method.

AIMS AND OBJECTIVES

1. Pharmacognostical and Preliminary Phytochemical Analysis of *Asana (Pterocarpusmarsupium*).
2. To evaluate Immunomodulatoy activity of *Asana* (*Pterocarpusmarsupium*) an in vitro method.

MATERIAL AND METHODS

Study design and setting: The study was paraclinical laboratory- based in vitro study. It was conducted at BLDEA'S SSM college of Pharmacy, Vijayapur.

Sample collection and Identification: The heart wood of the Asana (*Pterocarpu smarsupium Roxb.)* Was collected from college garden and the drug was authenticated by Dr V. S. Pujari mam, associate Professor, Department of Dravyaguna, BLDEA'S AVS Ayurved Mahavidyalaya, Vijaypura, Karnataka.

Processing and Extraction: the heartwood was collected and air dried in the shade. Then it was pounded into a course powder, using a mortar and pestle in department of Rashshatra-Bhaishjyakalpna, BLDEA'S AVS AMV Vijayapur. The extraction procedure was carried out at BLDEA'S SSM college of Pharmacy, Vijayapur.

Drugs: For NBT TEST Phosphate buffer saline was used as control and Lipopolysaccharid was used as standard. For Neutrophil locomotion and chemotaxis test the Casein was used as standard. candida albicans suspension was used in Phagocytosis and candidacidal assay. The NBT solution, Haematoxylin dye, Sabouraud broth, Hank's balanced salt solution, Giemsa stain were used in the study. All chemicals and reagents were used of analytical grade and were checked to ensure that they were not expired before the experimentation.

Determination of Phytochemical activity: The analytical study of drug *Asana (Pterocarpus marsupium)* is done under following headings

- Pharmacognostic study
- Physicochemical study
- Phytochemical study
- Qualitative analysis by Instrumental method

Determination of immunomodualtory activity

Asana (Pterocarpus marsupium) was subjected for screening of immuno-moduloatory activity with following parameters

- Nitrobluetetrazolium Test. (NBT)
- Neutrophil locomotion & Chemotaxis test.
- Phagocytosis and candidacidal assay.

A. Nitrobluetetrazolium Test

Nitroblue tetrazolium test was done according to the method described by Mali and Hatapakki, (2008) with minor modification. 7 Test tubes were taken for the test. Every test tube is filled with leucocyte suspension (5×10^6ml) in posphate buffer saline. 100 µl of phosphate buffer solution is added in to 1st tube and is taken as control. 2nd tube is added with 100 µl of lipopolysaccharide and is taken as standard. The remaining tubes were added with 100 µl of different concentration (6.25 µg/ml, 12.5 µg/ml, 25 µg/ml, 50 µg/ml and 100 µg/ml) of extract. All seven tubes were further added with 200 µl of 0.15% NBT solution. Now these were incubated for 20min at 37^0c. After incubation the tubes were centrifuged for 3-4 min at 400 and supernatant were discarded Further cells were treated with small volume of phosphate buffer saline solution and thin film was made with the drop on the clean glass slide. The slides were then dried, fixed by heating and were counter stained with carbol-fuchsin for 15sec. The percentage of NBT positive cells with blue lumps or granules were determined by observing tha stained slides for blue colour cells/lmps/granules under 40X objective for 200 cells.

B. Neutrophil Locomotion and Chemotaxis Tests

Neutrophil locomotion and chemotaxis test will done according to the method described by Mali & Hatapakki, 2008. Neutrophil cell suspension (10^6/ml) was prepared in phosphate buffer saline (PBS).The test samples, standard and control were be filled into the lower compartment (beaker) of the chemotactic chambers. Like chamber 1 was added with PBS and was used as control. Chamber 2 was added with casein and was used as standard. The remaining chambers were filled with different concentration (6.25 µg/ml, 12.5 µg/ml, 25 µg/ml, 50 µg/ml and 100 µg/ml) of the test plant extracts. The bottoms of the upper compartment (syringe) were placed with wet filter paper of 3 mm pore size and the upper compartments were filled with predetermined concentration of neutrophil cell suspension. These upper compartments were then placed into the lower compartments and were incubated for 180 min at 37^0c. After incubation then neutrophil suspension will empty by inverting the upper compartments and the filter papers was removed. The lower surfaces of the filter papers of all the chambers was fixed with ethanol and stained with haematoxylin dye for 5 min**.** The neutrophil locomotion and chemotactic abilities of test extracts were determined by observing the lower surface of stained filter papers under 100X oil emersion objective and numbers of neutrophil cells reached the lower surface of filters were counted.

B. Phagocytosis and Candidacidal assay

Phagocytosis of Candida albicans test was carried out according to method described by Ponkshe & Indap, 2002. Briefly, by using finger prick method human blood was added onto the clean glass slide and will incubated at 37^oC for 25 min for clotting. Normal sterile saline was further used to remove the clot, in such a way not to wash the adhered neutrophils. 100 µl of different concentration (6.25µg/ml, 12.5µg/ml, 25µg/ml, 50 µg/ml and 100 µg/ml) of test plant extracts was added on to adhered neutrophils and was incubated at 37^0C for 15 min. This was followed by addition of predetermined concentration of Candida albicans suspension and incubated at 37^0 C for 1 h. The slides were then drained, fixed using methanol and were stained with giemsa stain. The number of Candida albicans cells phagocytosed by a human neutrophil on the slide was determined microscopically using morphological criteria. The number of candida cells phagocytosed or engulfed by a

neutrophil was taken as Phagocytic index (PI) and the study as performed in triplicates.

Immunostimulation was calculated in percentage using the following equation.

Immunostimulation % = PI (samples) -PI (control) / PI (control) x 100.

Where, PI of samples: Phagocytic index of the test plant extracts, PI of control Phagocytic index without the test plant extracts(i.e., normally by neutrophils).

OBSERVATIONS AND RESULTS

Result of Phytochemical activity

- **Pharmacognostic analysis result**

1. Morphological study

Colour- red or yellowish brown
Odour- Specificodour
Taste-Astringent
Shape- variable size and thickness
External features- The pieces are angular, glistening.
Fracture-Vitreous

2. Microscopic features

The transverse sections of the stem bark were taken, cleared with clearing agent and mounted in glycerine water. The presence of cells consisting of lysigenous cavities, present in a row just below cork. Secondary phloem is found to be observed more space of the thickness of bark which consisting of sieve elements, phloem parenchyma. Parenchyma found merged towards the middle and outer side of phloem. Phloem fibres single usually numerous in groups forming alternating bands throughout phloem region wich is thick-walled and lignified with a small lumen also calcium oxalate which are rhomboidal crystals found scattered throughout the region, lysigenous cavities and tanniniferous ducts filled with red colour masses distributed throughout phloem region are also observed.

3. Organoleptic Study

Organoleptic parameters of asana (*Pterocarpus marsupium* Roxb.)

Characters	Heartwood of Asana
Colour	Red/ Yellowish brown
Smell	Specific odour
Sound	-
Taste	Astringent
Touch	Rough

- **Physicochemical analysis result**

Table no 1: Physicochemical Characters of Asana (Pterocarpus marsupium).

Sl No	Physicochemical Properties	Results (%w/w)	API Standard
1	Moisture content	4.05	-
2	Total ash	10.5	Not more than 18
3	Acid insoluble ash	1.34	Not more than 1.5
4	Water soluble ash	3.5	-
5	pH value	7	-
6	Alcohol soluble extractive	30.1	Not less than 7.5
7	Water soluble extractive	20.8	Not less than 11.5

- **Phytochemical analysis result**

Table no 2: Shows Inorganic constituents of Asana.

Sl no.	Inorganic constituents	Asana
1	Calcium	+
2	Magnesium	+
3	Sodium	-
4	Potassium	+
5	Iron	+
6	Sulphate	+
7	Phosphates	-
8	Chlorides	+

Table no 3: Shows Organic constituents of Asana.

Sl no	Tests	Alcohol	Aqueous
1	Alkaloid	+	+
2	Flavonoid	+	+
3	Saponoins	+	+
4	Glycoside	+	+
5	Triterpenoids	+	+
6	Tannin	+	+
7	Proteins	+	+
8	Carbohydrate	+	+
9	Steroids	-	-
10	Phenol	+	+

- **Qualitative analysis result**

1. TLC RESULT

TLC photo documentation, Rf values of Asana (*Pterocarpus marsupium)* alcoholic and aqueous extractare presented in respective tables and figures.

TLC plate of Asana (*Pterocarpus marsupium*) Figure 1

Under white light

At UV 254nm

Post derivations

Illustration of Rf values of phytochemicals separated during TLC from 50 % alcoholic ext. Of Asana with solvent system n-Butanol: Acetic Acid: Water (4:1:5) shows 2 spots at rf value 0.63 and 0.70 all brown spots.

Table no. 4: TLC Results of Alcoholic extract of Asana (*Pterocarpus marsupium*).

Under white light	At UV 254mm	Post derivation
0.63 Brown	-	0.63 Brown
-	0.70 Brown	0.73 Brown

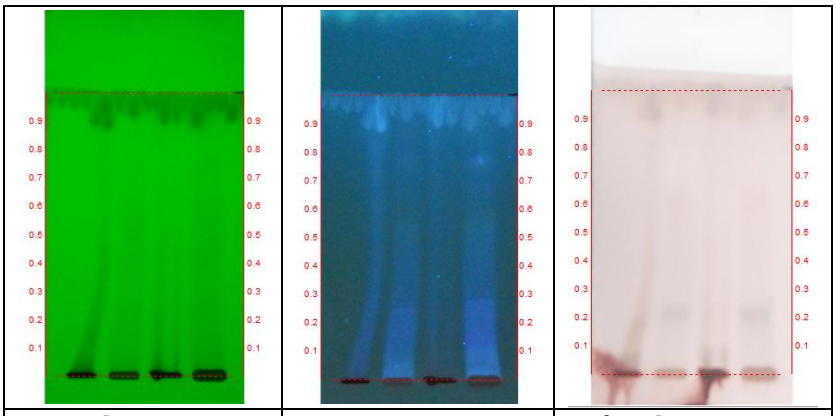
Showing Rf value of aqueous extract of Asana (Pterocarpus marsupium)with solvent systemn-Butanol : Acetic Acid: Water (4:1:5) shows 1 spots at Rf value at 0.60. On exposure to Iodine vapour one spots appear at Rf 0.76 brown.

Table no 5: TLC Results of Aqueous extract of Asana (*Pterocarpus marsupium*).

Under white light	At UV 254mm	Exposure to iodine vapour	Post derivation
-	0.60 Brown	-	0.60 Brown
-	-	0.76 Brown	0.76 Brown

2. High Performance Thin Layer Chromatography

HPTLC photo documentation, Rf values and densitometric scan of Asana (*Pterocarpus marsupium)* alcoholic and aqueous extractare presented in respective tables and figures.



Short UV | **Long UV** | **After derivatisation**

Figure 2: HPTLC photo of ethanol and aqueous extract of *Pterocarpus marsupium*

Solvent system – Diethyl ether: Toluene: Acetic acid (10.0: 1.0:10.0)
Track 1 – Ethanol extract of *Pterocarpus marsupium* – 3µl
Track 2 – Aqueous extract of *Pterocarpus marsupium* – 3µl
Track 3 – Ethanol extract of *Pterocarpus marsupium* – 6µl

Track 4 – Aqueous extract of *Pterocarpus marsupium* – 6µl

Table 6: R_f values of sample.

Short UV		Long UV		After derivatisation	
Ethanol extract of Asana (*Pterocarpus marsupium*)	**Aqueous extract of Asana (*Pterocarpus marsupium*)**	**Ethanol extract of Asana (*Pterocarpus marsupium*)**	**Aqueous extract of Asana *(Pterocarpus marsupium)***	**Ethanol extract of Asana *(Pterocarpus marsupium)***	**Aqueous extract of Asana *(Pterocarpus marsupium)***
-	-	-	-	-	0.22 (Pink)
-	-	-	0.27 (F. blue)	-	-
-	-	-	0.54 (F. blue)	-	-
-	-	-	-	-	0.62 (Pink)
-	-	-	0.75 (F. blue) Pterostilbene	-	-

*F-fluorescent

OBSERVATION AND RESULTS

1. Nitrobluetetrazolium

The cells are exposed to the yellow dye NBT, unstimulated neutrophils do not ingest this dye, but if they are stimulated to phagocytic activity, they take the dye into phagosomes and intracellular reduction of dye converts it to an insoluble, blue crystalline form (formazan crystals). These blue crystals are visible in the light microscopes and be counted. The NBT – test gives information about phagocytic function, since the dye is not taken into cells except by phagocytosis.

6.25, 12.5, 25,50and 100µg/ml concentration of Asana (*Pterocarpus marsupium*) were taken for study. 200cells per slide were observed for out of them only blue colored, stimulated to phagocytic activity were counted. The values were recorded in table.

Table 7: Nitroblue Tetrazolium (NBT) test of aqueous, alcoholic extracts on human neutrophils.

Normal control	Endotoxin activated plasma (positive control) 1 mg/ml	Concentration (µg/ml)	Aqueous extract	Alcoholic extract
		Mean no. of neutrophils per field		
		100	62	32
		50	57	35
17 ±0.0	89 ±0.0	25	38	19
		12.5	54	31
		6.25	53	36
		Mean ± SEM	52.80 ±4.02*	28.80 ±2.76**

*P < 0.01, **P < 0.001- as compared to positive control using one way ANOVA followed by Dunnett's 't' test.
S.E.M. – Standard error of mean

Neutrophil Locomotion And Chemotaxis

When the cells are placed in a gradient of chemoattractant, the cells change shape as they orient and migrate in unison towards the source of stimulus, a process called as "chemotaxis". Chemotaxis is assessed by measuring the movement of cells up a concentration gradient of stimulus. Most of neutrophil locomotion assess the behaviour of a population of cells moving through cellulose nitrate filters or under agarose. The cells are allowed to move a set time period then fixed, stained and assessed. In neutrophil locomotion and chemotaxis test, number of neutrophil cells reached the lower surface of filters were counted and recorded in table.

Table 8: Neutrophil locomotion and chemotaxis of aqueous and alcoholic extracts on human neutrophils.

Normal control	Casein (positive control) 1 mg/ml	Concentration (µg/ml)	Aqueous extract	Alcoholic extract
		Mean no. of neutrophils per field		
		100	92	150
		50	88	142
13 ±0.0	195 ±0.0	25	81	131
		12.5	76	124
		6.25	72	118
		Mean ± SEM	81.80 ±3.69**	133 ±5.83**

**P < 0.001- as compared to positive control using one way ANOVA followed by Dunnett's 't' test

Phagocytosis and Candidacidal Assay

The natural immunomodulator act by process of opsonization that is they neutralize the negative charge on candida cells thus making the initiation of phagocytosis. In candidacidal assay neutrophils were exposed to candida albiican suspension and incubated for 30 min for allowing candidacidal activity. And then sodium deoxycholate and Methylene Blue were added. Sodium deoxycholate lyses the leucocytes so only dead candida can be observed. Methylene blue is used for staining of dead candida cells. Using an improved Neabauer counting chamber the proportion of dead cells i.e. those which have taken up the methylene blue can be determined. The number of candida albicans engulfed per neutrophil was noted and recorded in table.

Table 9: Phagocytosis of killed candida albicans for aqueous and alcoholic extracts on human neutrophils.

Normal control	Human serum (positive control)	Concentration (µg/ml)	Aqueous extract	Alcoholic extract
Mean particle number				
2 ±0.0	6 ±0.0	100	2	3
		50	2	2
		25	2	2
		12.5	1	1
		6.25	1	1
		Mean ± SEM	1.33 ±0.33**	1.50 ±0.43**

***P* < 0.001- as compared to positive control using one way ANOVA followed by Dunnett's 't' test.

DISCUSSION

Immunomodulation is the process of modifying an immune response in a positive or negative manner by administration of a drug or compound. These are biological or synthetic substances, which can stimulate, suppress or modulate any of the immune system including both adaptive and innate arms of the immune response. Clinically immunomodulators works in three different ways. Immunoadjuvants- these are substances that enhance the production of antibodies along with antigen.Immunostimulants - these are substance (drug or nutrients) that stimulate the immune system by inducing activation or increasing activity of any of its components. They envisaged to enhance body's resistance against infection. Immunosuppressants - these are agents or antirejection medications are drugs that inhibit or prevent activity of immune system.

Ayurveda conceives a unique concept of Ojas, which is the essence of all the Dhatus and is responsible for vital strength of the body and resistance against the disease. Rasayana is believed to promote the process of Dhatuposhana and enrich Ojas leading to Vyadhikshamatva. The Rasayana herbs seem to exert their effect through immunosuppressant, immunostimulant and immunoadjuvant activities.

As Asana (Pteocrapus masupium) drug is having Kshaya, Tikta rasa, Ushna virya, Laghu guna and Katu vipaka and Rasayana effect. Its mechanism can be considered as follows

1. Due to its Kashaya, Tikta rasa, Ushna Virya, Laghu guna and Katu vipaka may be acting at level of Agni (Jatharagni and other agni's) by improving Agni means digestion, metabolism. They enhance the Agni and increase metabolism and help dhatu's for nourishment,create good quality of Saptdhatu.
2. Due to Ushna virya, Laghu guna and Katu vipaka may act on Srotas by promoting the competence of Srotas means microcirculatory channels in body and cleanses channels which leads to circulation of rasa easily in the body, thus it nourishes the Sapta Dhatu.

DISCUSSION ON PHYTOCHEMICAL STUDY

Prepared extracts was subjected for all the preliminary phyto-chemical studies, inorganic and organic tests. The results shows presence of Alkaloids, Flavonoids, Glycoside, Titerpenoids, Tannins, Proteins, Carbohydrate, Saponins and Phenols in both the extracts of Asana*(Pterocarpus marsupium*).Inorganic tests shows presence of Calcium, Magnesium, Potassium, Iron, Sulphate, and Chloride in the drug extract.

Literature survey indicates that flavonoids are possesses wide variety pharmacological activities like antioxidative, anticarcinogenic, antiviral, anti-inflammatory, antibacterial and immune stimulating effects.[4]

Literature survey indicates that saponins possesses wide variety pharmacological activities like antiallergic, cytotoxic, immunomodulating, antitumor, antiviral and antifungal properties. Recently pterostilbene isolated from *Pterocarpus marsupium has* been reported for immunostimulating activity.[5] Tannins are also known to possess immunostimulating activites, Triphala containing *Terminalia chebula, Terminalia belerica*, and *Emblica officialis*, which are rich in tannins, has been reported to have immunostimulating activity.[6-8]

The qualitative analysis of Asana (Pterocarpus marsupium) were done by Thin layer chromatography and High performance thin layer chromatography. The Result of HPTLC shows there is presence of Pterostilbene in the aqueous extract of Asana (Pterocarpus marsupium). Literature survey indicates that Pterostilbene possesses multiple benefits in the

treatment and prevention of human disease like Immunomodulatory activity[9] antioxidant activity, anti-inflammatory activity, anticarcinogenic properties leading to improved function of normal cells and inhibition of malignant cells.[10]

Evaluation of Immunomodulatory activity

As a part of immunomodulatory activity the following in vitro methods were performed on human neutrophils to assess the activity of aqueous and alcoholic extracts of heart wood of Asana(Pterocarpus marsupium) at the concentration range of 6.25, 12.5, 25,50and 100µg/ml**.**

1) Nitroblue tetrazolium (NBT) Qualitative Test

The results obtained indicate that the aqueous extract of heart wood of Asana(Pterocarpus marsupium) has stimulated the neutrophils to the phagocytic activity to the extent of 60-70% whereas the alcoholic extracts stimulated the activity to the extent of 40% at all the concentration range as compared to standard endotoxin, which was significant ($p<0.0001$). It is also significant when compared to control.

However, on higher dilution with the phosphate buffer (i.e.at low concentration) showed good results. This may be due to purer drug available on dilution, which has the potent activity.

2) Phagocytosis and Candidacidal assay

The results obtained indicate that the alcoholic extracts of heartwood of Asana (Pterocarpus marsupium) rather than aqueous extract of stem have stimulated the phagocytosis of killed Candida albicans more. The mean particle number (MPN) were 2 when compared to 6 of standard (positive control) and 1-2 of control at the concentration range.

3) Neutrophil Locomotion and Chemotaxis

The results show that the alcoholic extract of heartwood of Asana (pterocarpus marsupium) significant chemotaxis than the aqueous extract when compared to standard Casein. It is also significant when compared to control ($p<0.0001$). It is meant that purer drug, which is devoid of chlorophyll and other fatty material may be available, which in turn give the increased activity rather than the higher concentrations.

Asana (pterocarpus marsupium) the alcoholic and aqeous extract shows significant immune-modulatory effect. As the test were conducted mainly on polymorphoneutrophils. It can be predicted that Asana (pterocarpus marsupium) improves non-specific immunity.

From above results of immune-modultory assay with three parameters it is noted that the extract of heart wood of Asana (pterocarpus marsupium) shows significant immune-modulatory activity.

CONCLUSION

- The Immunomodulatory activity of Asana (*Pterocarpusmarsupium*) is probably due to presence of chemical constituents like flavonoids, tannins, alkaloids, saponnins.
- Asana (*Pterocarpusmarsupium*) was subjected for immunomodulatory activity with three parameters. Results of all three parameters shows significant immunomodulatory effects.
- NBT- all the concentration of Asana (*Pterocarpusmarsupium*) shows significant stimulation of neutrophil for phagocytic activity.
- Phagocytosis - Asana (*Pterocarpusmarsupium*) significantly stimulated neutrophils in blood samples to ingest candida albicans, thus promoted the phagocytosis.
- Neutrophil locomotion and Chemotaxis - Asana (*Pterocarpusmarsupium*) significantly stimulated neutrophil in blood samples to migrate for neutrophil locomotion and chemotaxis test.
- The alcoholic extracts shows the significant results in case of Nitrobluetetrazolium and Neutrophil locomotion and chemotaxis and in case of aqueous extract shows significant results in Phygocytosis and candidacidal assay.
- The result of present study indicated that the Asana (*Pterocarpusmarsupium*) is having Immunomodulatory activity.

SUMMARY

Asana (Pterocarpusmarsupium) was selected as a drug of choice for this study, because in Bhavaprakasha Nighantu referance is found regarding Immmunomodulatory activity of Asana. The results shows presence of Alkaloids, Flavonoids, Glycoside, Titerpenoids, Tannins, Proteins, Carbohydrate, Saponins and Phenols in both the extracts of Asana*(Pterocarpusmarsupium*). At the end of study the results showed the significant results of immmunomodulatory activity of Asana (Pterocarpusmarsupium). The alcoholic extracts shows the more potent than aqueous extract.

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