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# A NOVEL IMMUNOMODULATORY ACTIVITY OF *SIDAGLUTINOSA*-A MAGICAL INDIAN TRADITION MEDICINE

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**ABSTRACT**

Plants are the immense resource of nutrients, therapeutics, immune boosters and anticancer etc. Medically important plants are crucial role players in preventing and curing diseases and disorders. In present study, the ethanolic extract of plant *S. glutinosa* was evaluated for immunomodulatory activity using various *invitro* and *invivo* immunomodulatory models. The extract was subjected for isolation, purification and structural characterization of pure fraction 2 using various analytical techniques. Invivo studies indicated increased cell mediated immunomodulatory activity evidenced by increased phagocytic index *i.e.*, 0.011±0.0003 in Swiss Albino mice orally administered with 200mg/kg/bw dose of ethanolic extract. Dose dependent increase of WBC and time dependent DTH response was noticed in mice treated with high dose of the extract in contrast to control groups. Humoral immune response elicited by 100mg/kg/bw and 200mg/kg/bw of ethanolic extract which showed increased Hemagglutination titer 7.5±0.34. The TNF-α and IL-6 level were found to be 21.5 ± 3.0 and 29.5 ± 0.68pg/ml in mice treated with ethanolic extract of 200mg/kg/bw which strongly supports the immunomodulatory role of ethanolic extract when compared to diseased control. A fraction 2 of ethanolic extract of *S. glutinosa* purified through column chromatography showed single peak at 254nm with RT 1.3minute and a molecular mass of 476.07 Daltons when analyzed by LCMS. $^{1}H$ and $^{13}C$ NMR spectrum revealed molecular formula $C_{25}H_{16}O_{10}$. The fraction 2 was further studied for *Invitro* immunomodulation study on RAW 264.7 macrophage cell line which indicated proliferation of RAW 264.7macrophage cell lines and elevated level of TNF-α and IL-6 gene expression.

## INTRODUCTION

Plants and microorganism are the unsurpassed sources of most of therapeutic biopharmaceuticals exploredin health care to treat diseases. World Health Organization (WHO) has enlisted around 21,000 medically important plants used throughout the globe. Approximately 2500 plant species discovered in India out of which 150 species are commercially used in pharmaceutical industries for pilot scale production of mainstream drugs [1].It is well known fact that herbal based treatment of diseases is not recent origin and from ancient time plants and their extracts is continuously applied to get rid of many

disorders and diseases. Plants can be used as biological weapon against wide range of diseases as herbals constituents reveals both nutritional and therapeutic properties like broad spectrum biological activities and most of the illness can be efficiently managed by treating them with herbal products [2]. Phytochemical constituents of plants have been reported for various activities like potential antimicrobial activity against variety of microorganisms, anti-inflammatory and anti-arthritis activity [2, 3]. It has been estimated that, more than 60% of anticancer drugs are of plant origin [4]. Additionally, many Indian medicinal plants are exhibited immunomodulatory activity that includes immunoadjuvants, immunostimulants and immunosuppresants [5]. The ethanolic extract of *Sonerilatinnevelliensis* evaluated for immunomodulatory activity at the dose of 400mg/kg body weight revealed significant increase in the white blood count and delayed type hypersensitivity response[6].The evaluation of ethanolic extract of herb, *Galiumaparine* L. a dietary supplements and folk remedies for immunomodulatory activities revealed significant increase in activity of immunocompetent blood cells at 96% of extract tested [7]. The family Malvaceae consists of 244 genera and 4225 species that encompass shrubs, trees and herbs. Genus *Sida* L. consists of herbs are traditionally used for treatment of wide array of ailments like inflammations, tuberculosis, diarrhea, urinary infection, respiratory problems like asthma, bronchitis, miscarriages, cardiac ailments, skin problems and neuronal associated problems [8]. In addition, the root extracts of *Sida acuta* Burm. f. and *Sida* ***humiliscav,*** revealed immunomodulatory activities in Swiss Albino mice and Kwath and **choorna prepared from roots** *Sida* ***humiliscav,***inc**.** showed immunomodulatory activity evidenced by increase in white blood count and thickness of **foot pad of mice [9, 10].**The root of *S. cordifolia* L. popularly known as 'Bala' (*strength* in Sanskrit) is also reported for its immunostimulatory activity in Wister rat and (10E, 12Z)-9-hydroxyoctadeca-10,12-dienoic acid purified from the whole plant *S. cardifolia* found to be first natural anti-HIV-1 agent that inhibited the replication of HIV-1[11].Hydroalcohalic extract of leaves and stems of *Sida spinosa* Linn. also tested in swiss albino mice revealed immunomodulatory activity. The extract orally administered at 200 and 400mg/kg/day till 21days indicated significant increase in the primary and secondary immune response and leukocytes count and phagocyte index [12]. But as per our literature survey there are no report of immunomodulatory activity of *S. glutinosa* and the present study is the first report on immunodulatory activity of *S. glutinosa*. Present study is the extension of our previous work [13]. The ethanolic extract of *S. glutinosa*was investigated for its immunomodulatory potential by *invitro* and *invivo* immunomodulatory models. Further, pure fraction 2 from ethanolic extract was purification of by silica gel column chromatography and its structural elucidation was carried using various analytical tools like TLC, LCMS and NMR.

## MATERIALS AND METHODS

**Preliminary work:** The preliminary work on the plant *Sida glutinosa* that includes collected, authentication, phytochemical screening, antioxidant activity and flavonoids estimation was published in previous article [13]. Since, ethanolic extract was revealed significant antioxidant activity and flavonoids content hence it was selected for further investigation of its immunomodulatory activity by using *in-vitro* and *in-vivo* models. Structural elucidation of pure fraction purified from the ethanol extract of *S. glutinosa*also carried out. .

### *IN VIVO* IMMUNOMODULATORY STUDY

**Cell Mediated Immunity**

**Carbon clearance test and Complete blood count:** Immunomodulatory activity of ethanolic extract of *S. glutinosa* was investigated using *invivo* experimental models *i.e.,* a carbon clearance test and complete blood count technique an [14].The carbon clearance test consists of five groups of Swiss albino mice and each group consists of six animals. All animal body weight were recorded and Group-I, II and III mice were treated with 1ml of 1% CMC, Levamisole hydrochloride(positive control) at 50mg/kg/bw, and Cyclophosphamide (negative control) at

200mg/kg/bw respectively. The group IV and V mice were orally administered with ethanolic extract of *S. glutinosa* at lower dose 100mg/kg/bw and higher dose 200mg/kg/bw. The test samples orally given for 5 days and normal control for 10 days and at the end of fifth day, after 48 hours, mice were injected via the tail vein with carbon ink suspension of 10µl/gm.bw. Blood samples were drawn from the retro-orbital vein at 0 and 15 min, 25 µl sample was mixed with 0.1% sodium carbonate solution (2 ml) and its absorbance at 660 nm was determined. The phagocytic index **K** was calculated using the following equation:

$$K = \frac{\text{Log}_e OD_1 - \text{Log}_e OD_2}{15}$$

Where, $OD_1$ and $OD_2$ are the optical densities at 0 and 15 min, respectively

Complete blood count technique was conducted by continuing same treatment used in carbon clearance test14 days using same dosage. On the 14th day, 2ml of fresh blood is drawn by retro-orbital puncture from each animal and analyzed for complete blood count and differential blood count study.

**Delayed type of hypersensitivity (DTH):**

DTH was studied in Wistar Albino rats and grouping and dosing is maintained same as explained in carbon clearance test. All groups of rats were primed by subcutaneously injecting 0.1 ml of suspension containing $1\times10^8$ SRBC into the right hind footpad. The contra-lateral paw also received an equal volume of 0.1% phosphate buffered saline (PBS). After 14th day administration of extracts, the animals were challenged by subcutaneously injecting 0.1 mL of $1\times10^8$ SRBCs into the left hind footpad of the rats. The extent of delayed-type hypersensitivity (DTH) response in the rats was determined by measuring the footpad thickness after 4, 8, and 24 h of challenge using vernier calipers. The difference in the thickness of the right hind paw and the left hind paw was then used as a measure of DTH reaction and was expressed as a mean percent increment in thickness/edema [15]**.**

DTH = Left footpad challenged with antigen– Right foot pad control X 100

Left footpad challenged with antigen

**HUMORAL IMMUNE RESPONSE**

**Hemagglutination (HA) titer method**

The effect of ethanolic extract on humoral immune response was studied in Swiss albino mice. The grouping of mice and dosing were maintained as mentioned in the carbon clearance test. All rats of each group were immunized with $0.5 \times 10^9$ cells/0.1 ml of SRBCs at day 0 (Note: Sheep erythrocytes (SRBC) were prepared using the published protocol [20].All the groups were intra-peritoneally treated for seven consecutive days after which blood sample collected from the tail and antibody titer was determine from serum using HA titer method. In 96-well plates, 25 µl of PBS was added in all the wells, except the last column which was denoted as control. In the first row of micro titer plate, 25 µl serum was placed and 2-fold serially diluted up to the 8th row of the micro titer plate. Twenty-five microliters of 10 v/v SRBCs were then dispensed in micro titer plate which was incubated at 37°C for 1 hour and after incubation HA titer value was determined [16].

**Determination of rat interleukin-6 (IL-6) and Tumor Necrosis Factor-α (TNF-α) by ELISA**

To evaluate the effect of ethanolic extract of *S. glutinosa* on TNF-**α** and IL-6, the control animals were treated with vehicle orally for 10 days and test samples were given orally for 14 days after which blood samples collected from the retro-orbital vein and serum separated was used for the estimation of IL-6 and TNF-α was carried out by ELISA Kit method supplied by Krishgen.

**PURIFICATION AND CHARACTERIZATION OF PURE METABOLITE**

TLC was conducted for ethanol extract (10mg/ml) of *S. glutinosa* using solvent system chloroform: methanol (8:2) and Relative frequency ($R_f$) value were determined. Ethanol extract (10grams) was loaded in silica gel column and initially eluted with hexane, followed by various ratio of hexane and chloroform. Finally washing was done using chloroform and methanol at various concentrations. At each elution, 20ml of eluate was collected and the purity was cross checked by conducting TLC and elutes having similar

fractions were pooled down. Since the fraction 2 obtained at hexane: chloroform (50:50) revealed significant immunomodulatory activity, hence the fraction was selected for further study..

**LCMS AND FTIR:** A pure fraction 2 dissolved in methanol at 1mg/ml concentration and 5µl of 1mg/ml solution was injected into the column. The fraction was run for total of 5 minutes and detection was carried out at 254nm, followed by the by the detected in mass spectrophotometer. Both the parental and other daughter fragments were detected. Based on the fragmentation pattern, the category of the compound was detected. To determine the functional groups, the fraction 2 was mixed with KBr pellets and mixture was made into round pellet block and scanned from 4000 $cm^{-1}$ to 500$cm^{-1}$ wavelength. The various peaks of FTIR were observed and corresponding functional groups were detected.

**$^{13}$C and $^{1}$H NMR:** The pure fraction 2 was dissolved in deuterated solvent and subjected for analysis by NMR. Various peaks obtained during the analysis were captured. From the analysis of NMR peaks total carbon and hydrogen atoms in the pure fraction were calculated.

### *IN VITRO* IMMUNOMODULATORY STUDY

The pure fraction 2 purified from ethanol extract of *S. glutinosa* was evaluated for *invitro* immunomodulatory activity.

#### Cytotoxicity of fraction 2 on mammalian cells

*Invitro* Cytotoxicity of fraction 2 on RAW 264.7 macrophage cell lines plated in 96 well microtitre plate at 2 x $10^5$ cells/well using Dulbecco's modified Eagle medium (DMEM) was tested. To the DMEM 10% fetal bovine serums, gentamycin at 50 µg/ml were added and plate was incubated at 37C° and 5% $CO_2$ for 2 hours. The fraction 2 was added to the plate at 500, 250, 125, 65.5 and 31.25µg/ml concentration and experiment was incubated at 37C° for 72 hours. After incubation, the 20µl/well of MTT dye was added to all wells and readings were recorded at 570nm.Experiments were conducted in triplicate [17].

**Cell proliferation assay:** The effect of pure fraction 2 of *S. glutinosa* on proliferation of RAW 264.7 cell line was determined by MTT assay.In 96 well microtitre plate, RAW 264.7 cell line were seeded at 5 × $10^5$ cells/ml using DMEM medium and plate was incubated at 37C° and 5% CO for 24hours. After incubation, cell lines were treated with pure fraction 2 at 500, 250,250, 62.5, 31.25, 7.81. 3.9 and 0.97µg/ml and incubation is continued for 24hours. To each well, 10µl of MTT dye (5mg/ml) was added and plate was incubated for 4hours at 37C. After incubation MTT dye was removed and 100µl of DMSO was added and absorbance at 570nm was measured [18].

**Measurement of cytokines:** Cytokines production *viz.,* IL-6 and TNF-α in RAW 264.7 macrophage cell lines treated with fraction 2 was determined by seeding the cell lines as explained in the cytotoxic assay. After incubation for 2 hours, in presence of fraction 2 and vehicle the cells were stimulated by LPS at 500ng/ml. For quantification of TFNα and IL-6, cell-free supernatants were collected after 4 hours and 24hours respectively and stored at -80˚ C. TFNα and IL-6 concentrations in supernatants estimated by ELISA, using ERBA kit according to the instructions provided by the manufactures [17].

## RESULTS AND DISCUSSION

### *IN VIVO* IMMUNOMODULATORY STUDY

**Carbon clearance test:** Cell Mediated Immunity of the ethanol extract of *S. glutinosa* evaluated by carbon clearance test revealed significant increase in the phagocytic index in contrast to control groups when extract was orally administered to the mice at low and high doses. The results were found to be statistically significant (*p<0.0001*) and the trend of experiment were comparable to the published data. Phagocytosis is the important defense mechanisms that protect the body from foreign bodies or pathogens like bacteria, fungi, toxin and other microbes. Ethanolic extract of *S. glutinosa* revealed significant potential in the enhancement of immune system as evidenced by phagocytic index

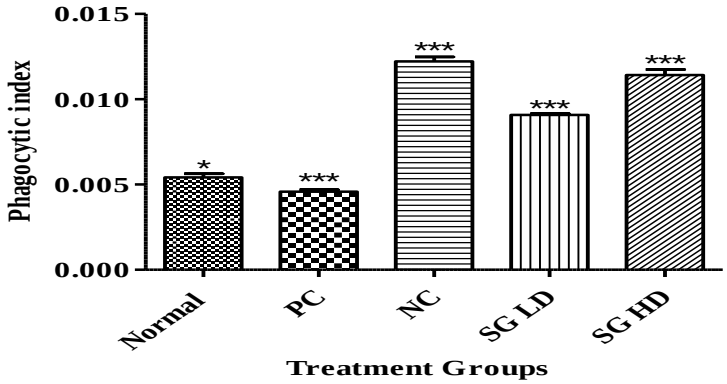


**Figure 1.Graphical representation of carbon clearance test.**(Note: PC- Positive control (Levamisole-HCl), NC- Negative control (Cyclophosphamide), SD-*S. glutinosa*, LD&HD-Low and High Dose

**Table 1.Effect of ethanolic extract of *S. glutinosa* on CBC mice**

| Treatment | Dose (mg/kg/bw) | Complete Blood Count | | |
|---|---|---|---|---|
| | | RBC ($10^6$/µl) | WBC ($10^3$/µl) | Hb (g/dl) |
| Normal saline (Vehicle) | Nil | 7.15±0.46 | 6308±63.79 | 14.86±0.42 |
| Cyclophosphamide (NC) | 200 | 6.11±0.19 | 1463±81.50 | 10.90 ±0.48 |
| Levamisole-HCl (PC) | 50 | 7.12±0.11 | 4973±73.64 | 13.23±0.34 |
| *S. glutinosa* low dose | 100 | 6.71±0.08 | 1855±22.57 | 11.63±0.09 |
| *S. glutinosa* high dose | 200 | 7.08±0.04 | 3781±59.00 | 12.10 ±0.38 |

Note: Statistically significance of data *n=3 was found to be RBC* (*P<0.02)*,WBC&Hb (*P<0.0001*)

**Table 2.Determination of Delayed type of Hypersensitivity**

| Treatment | Dose (mg/kg/bw) | Mean diameter of foot pad thickness (mm) | | |
|---|---|---|---|---|
| | | 24 hours | 48 hours | 72 hours |
| Normal saline (Vehicle) | Nil | 0.39 ± 0.004 | 0.35 ± 0.005 | 0.28 ± 0.007 |
| Cyclophosphamide (NC) | 200 | 0.19 ± 0.020 | 0.17 ± 0.020 | 0.15 ± 0.010 |
| Levamisole-HCl (PC) | 50 | 0.52 ± 0.030 | 0.36 ± 0.010 | 0.24 ± 0.010 |
| *S. glutinosa* low dose | 100 | 0.43 ± 0.010 | 0.34 ± 0.020 | 0.18 ± 0.003 |
| *S. glutinosa* high dose | 200 | 0.51 ± 0.007 | 0.35 ± 0.010 | 0.22 ± 0.006 |

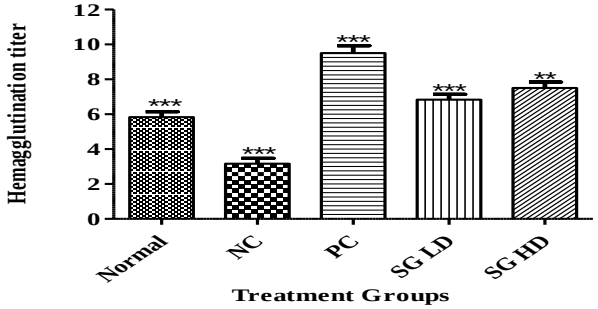


**Figure 2.Study of ethanolic extract doses of *S. glutinosa* on HA titer**
(Note: PC- Positive control (Levamisole-HCl), NC- Negative control (Cyclophosphamide), SD-*S. glutinosa*, LD&HD-Low and High Dose)

**Table 3.Determination of rat Tumor Necrosis Factor-α (TNF-α) by ELISA**

| Treatment | Dose (mg/kg/bw) | TNF-α (pg/ml) |
|---|---|---|
| Normal saline (Vehicle) | Nil | 07.02±1.3 |
| Cyclophosphamide (NC) | 200 | 26.05±0.28 |
| Levamisole-HCl (PC) | 50 | 15.93±1.1 |
| *S. glutinosa* low dose | 100 | 24.74±2.4 |
| *S. glutinosa* high dose | 200 | 21.5±3.0 |

**Table 4. Determination of rat IL-6 by ELISA**

| Treatment | Dose (mg/kg/bw) | IL-6 (pg/ml) |
|---|---|---|
| Normal saline (Vehicle) | Nil | 13.5±0.53 |
| Cyclophosphamide (NC) | 200 | 32.85±1.5 |
| Levamisole-HCl (PC) | 50 | 17.8±0.52 |
| *S. glutinosa* low dose | 100 | 29.5±0.68 |
| *S. glutinosa* high dose | 200 | 28.55±0.61 |

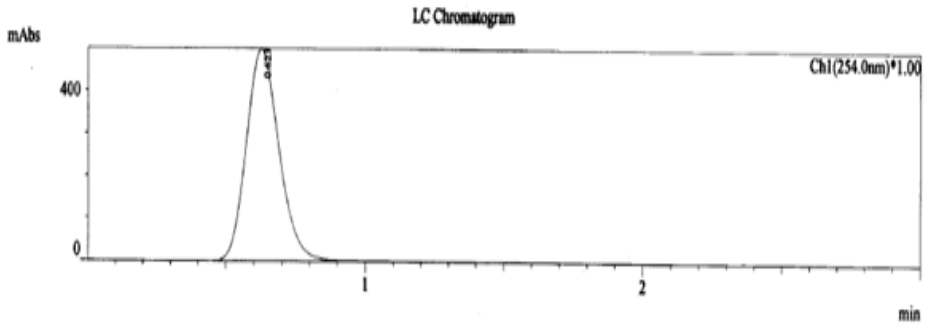


**Figure 3.Chromatogram of LCMS of fraction2 of *S. glutionsa***

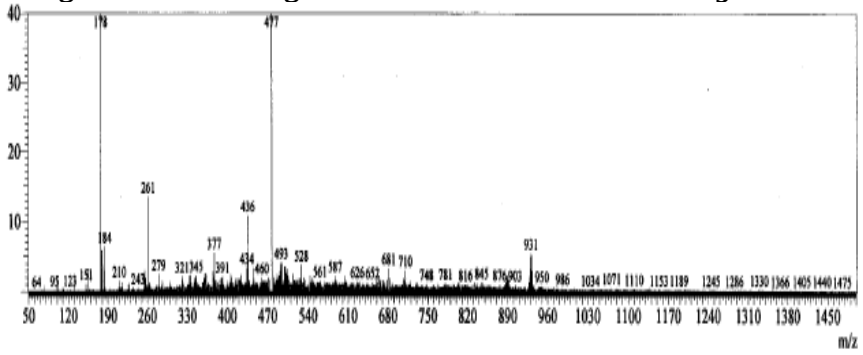


**Figure 4. Mass spectroscopy of fraction 2**

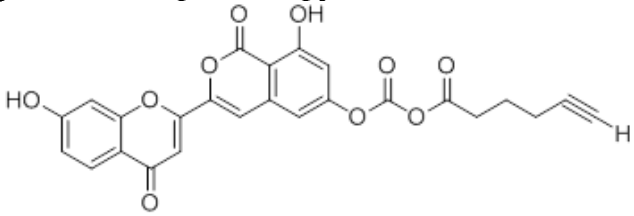


**Figure 5.The chemical structure of fraction 2 of *S. glutinous***

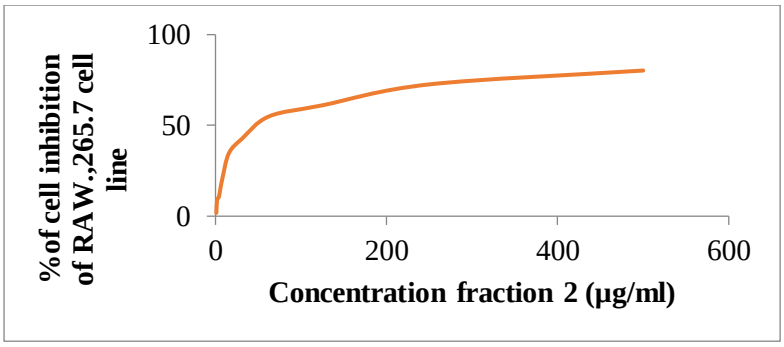


**Figure 6. Effect of fraction 2 on percentage inhibition of RAW 264.7 cell line**

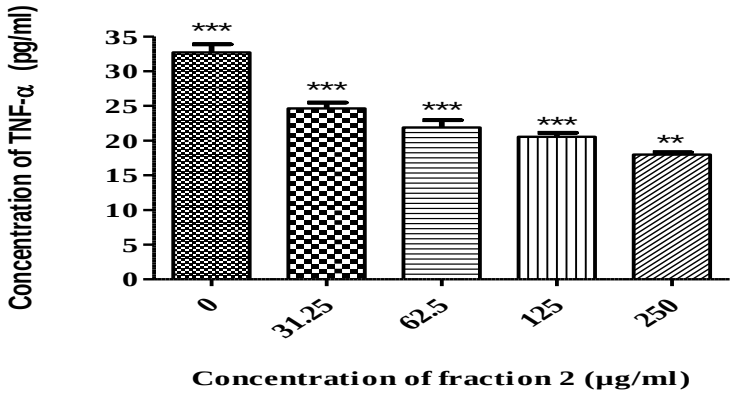


**Figure 7: TNFα profile of RAW264.7 treated with fraction 2**

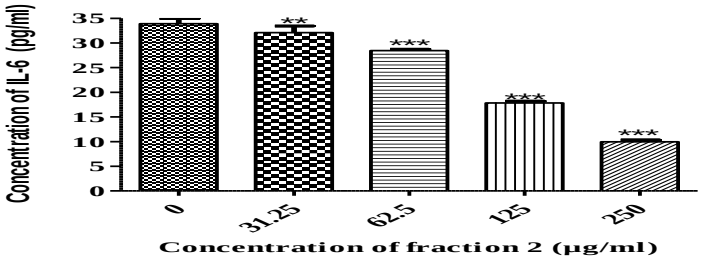


**Figure 8: IL-6 profile of RAW264.7 treated with fraction 2**

## RESULTS AND DISCUSSION

### *IN VIVO Immunomodulatory study*

**Carbon clearance test:** Cell Mediated Immunity of the ethanol extract of *S. glutinosa* evaluated by carbon clearance test revealed significant increase in the phagocytic index in contrast to control groups when extract was orally administered to the mice at low and high doses. The results were found to be statistically significant ($p<0.0001$) and the trend of experiment were comparable to the published data. Phagocytosis is the important defense mechanisms that protect the body from foreign bodies or pathogens like bacteria, fungi, toxin and other microbes. Ethanolic extract of *S. glutinosa* revealed significant potential in the enhancement of immune system as evidenced by phagocytic index.

### Complete blood count (CBC)

Blood cells *viz.*, white blood cells, play important role in human immune system that eliminate foreign body that enters into human body. Hematological parameters analysis of blood collected from animal groups treated with high dose of ethanol extract showed increased WBC count in comparison to low

dose of extract. The count of WBC for high dose of extract was found to be 3781 WBC x $10^3$/µl whereas as that of low dose it was 1855 x $10^3$/µl. Similarly, BRC count was also increased at high dose of extract as indicted in the Table 2. On the whole administration of ethanol extract of *S. glutinosa* elevated all hematological parameters in dose dependent manner and results were statistically significant (Table 1).

**Determination of Delayed type of Hypersensitivity**

DTH reactions directly mediated through sensitization of T lymphocytes, monocytes and macrophages by antigens are important component of immunity. Increase in the DTH is one of the strong indicators of immunomodulatory process. The response of DTH was evaluated in Wistar Albino rats after treating them with low and high doses of ethanolic extract of *S. glutinosa* and challenging them with SRBC antigens for 0, 24, 48 and 72 hours. Generally, increased DTH response was observed against all doses compared to the control groups(Table2) and all experimental data *n=3* were found to be significant at p<0.0001. Results indicated that the extract is strong immunomodulatory agent.

**HUMORAL IMMUNE RESPONSE**

**Hemagglutination (HA) titer method:**

While cellular mediated immune system is mediated through immune cells like T lymphocyte, monocytes and other leucocytes, humoral immune system is consist of antibodies which fight the foreign antigens like bacteria, fungi and other pathogens. Increase in the antibody titer is evidence for the immunomodulatory activity. In present investigation, Swiss albino mice treated with ethanol extract revealed higher level of antibody titer in mice treated with high dose of extract in contrast to low dose of extract. The HA titre value ethanol extract at high dose was found to be 7.5±0.34 whereas that of positive control was found to be 9.5±0.3 as shown in Figure 2. The statistically significance value of experimental data were to be *P<0.0001*. Based on the results one can conclude that extract can act as best immunomodulaotry agent.

**Determination of cytokines:** The TNF-α and IL-6 are important pro-inflammatory cytokines which plays very important physiological role in immune response and in inflammatory reactions. In present investigation concentrations of cytokines *viz.*, TNF-α and IL-6 in serum of animal treated with ethanol extract of *S. glutinosa* indicated dose dependent decreased in level of both cytokines. The level of TNF-α and IL-6 at 200mg/kg/bw of mice was found to be 21.5±3.0and 28.55±0.61pg/ml respectively, whereas the concentration of cytokines at 100mg/kg/bw was found to be 24.74±2.4 pg/ml of TNF-α and 29.5±0.68 pg/ml of IL-6 (Table 3 and 4**).**The group treated with cyclophosphamide has shown significant increase in the level of TNF--α (26.05±0.28)and IL-6 (32.85±1.5). Levamisol-HCl served as positive control. All mean values were moderately significant *p<0.001*.

**PURIFICATION AND CHARACTERIZATION OF PURE METABOLITE**

**TLC and silica gel column chromatography**

The ethanol extract separated by silica column chromatography and each 20ml of fraction collected and checked for purity. Similar fraction pooled down and fraction 2 yield was highest in concentration and also indicated significant immunomodulatory activity. Hence fraction 2 was subjected for further analysis.

**LCMS AND FTIR:**

The purity and molecular mass can be determined by LCMS. The fraction 2 of ethanolic extract of *S. glutinosa* analyzed by LC revealed single peak with RT of 1.3minutes detected at 254nm. The chromatogram indicated purity of the fraction 2 as evidenced by the single peak. The analysis of fraction 2 by mass spectroscopy indicated signal at m/z 477.07 m/z and also revealed the fragmentation peak 178, 177+1 m/z as indicated in the figure 4. Based on signals shown by the mass spectrum, the molecular formula of fraction 2 was found to be $C_{15}H_{16}O_{10.}$ Determination of functional groups of the metabolite is performed by the analysis of fraction 2 by FTIR.

Analysis of fraction 2 by FTIR revealed peak 3364.52 $cm^{-1}$ indicating present of OH group, 2954.63 $cm^{-1}$attributed to Ar-C=H- and 2112.62 $cm^{-1}$corresponding to -R-C=C-H- group. The fraction also indicated peak at 1736.74&1623.30 $cm^{-1}$that indicted Ar-CH-O-C-O-R- group and presence of group Ar-C=CH and Ar-mono substitution evidenced by the peaks 1461.11 $cm^{-1}$ and729.44 $cm^{-1}$ respectively.

**$^{13}$C and $^{1}$H NMR: Fraction 2 analyzed by 1H NMR revealed the signals at** Ha5H-δ7.2866-7.205, Haa1H- δ 7.93, Hb1H- δ 6.94, 1H-δ6.403123 6H δ-2.8708-2.796. Based on the signals the total number of hydrogen atoms was found to be 16. Carbon atoms of fraction 2 was analyzed by $^{13}$C NMR revealed signal at 166.19-163.66 corresponds to presence of carbonyl groups (=C=O). Peak at 136.43, 135.735 attributed to Ar-CH=CH and peak at 129.66, 129.57 is due to Ar-HC=CH group. The peaks at 111.41,111.35 corresponds to AR-CH=C, signal at 93.05 is attributed due to -$C^{=}$-CH- and signal at 30.98 is attributed to -$CH_2$-$CH_2$- groups and presence of -$CH_2$-$CH_2$ reflected at peak 25.48. **$^{13}$C** NMR signals revealed presence of 15 total number of carbon atoms in fraction 2. The structure of fraction 2 elucidated based on the results of MS and NMR was indicated in Figure 5.

## *IN VITRO* IMMUNOMODULATORY STUDY

**Cytotoxicity of fraction 2 on mammalian cells:** Fraction 2 of ethanol extract was tested at 500, 250, 125, 62.5, 32.25, 15.62, 7.81, 3.9, 1.95, 0.97 µg/ml for its cytotoxic activity. The fraction 2 revealed dose dependent cytotoxic activity on RAW 246.7 cell lines. CTC 50 value of fraction was found to be 146.88µg/ml

**Cell proliferation assay:** Immunomodulatory activity study of pure fraction 2 on RAW264.7 macrophage cell lines indicated decreased proliferation of the cell lines with respect to increased concentration of dose. No significant decrease in cell lines notices at the test concentration of 500µg/ml of fraction 2.

**Estimation of TNFα and IL-6:**

*In vitro* cell line based study is very important model for study immunomodulatory activity of plant metabolites. Determination important biomarker cytokines like TNF and IL in cell lines treated with plant metabolites is essential to show immunomodulatory potential of plant extracts. In present study cell supernatant of LPS induced RAW 264.7 cell line collected after the treatment by fraction 2 indicated the dose dependent elevation of concentration of TNF-α and IL-6 as indicated in Figure 7 and 8. Significant decrease in level of both cytokines indicated that fraction 2 of *S. glutinosa* is strong immuunomodulator.

**Statistical analysis:** All experiments were performed in triplicate (*n=3*) and results were expressed as Mean ± SD. The experiment data were analyzed using Graph pad Prism 5.All data were analyzed by One-way ANOVA. The Mean values of treatment groups were compared with control groups using Dunnett test at 95% confidence limit. The difference between the mean value of extract treated groups and control groups were considered significant when the $p < 0.001$.

**CONCLUSION:** Immunomodulatory potential of plant metabolite can be determined using various immunomodulatory models in which both cellular mediated immunity and humoral mediated immunity can be estimated. In the present investigation ethanol extract of *S. glutinosa* investigated for immunomodulatory activity in *invivo* and *invitro* models the crude extract exhibited increased concentration of immunomodulotors like phagocytic index, DTH and showed activity on cytokines like TNFα, IL-6 in an effective way. Immune response of fraction 2 of ethanol extract of *S. glutionosa* decreases cytokines concentration of TNF-α and IL-6 in *invitro* cell line based studies in a dose dependent manner. Increase in the concentration of immune mediated cells and antibody titer strongly supported the immunomodulatory activity of *Sida glutinosa.*.

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