Sterols possess immunomodulatory property: Activity guided isolation and in vitro screening of phytoconstituents of *Pongamia glabra* and *Ficus glomerata*

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**A R T I C L E   I N F O**

**Article history:**
Received 30 August 2019
Revised 8 November 2019
Accepted 24 December 2019
Available online 6 July 2020

**Keywords:**
activity guided isolation
*Ficus glomerata* Roxb.
immunomodulation
*Pongamia glabra* Vent.

**A B S T R A C T**

**Objective:** To isolate the phytoconstituents from the methanolic extracts of the stem bark of *Pongamia glabra* and *Ficus glomerata*, characterize spectroscopically and screen for *in vitro* immunomodulatory activity on human neutrophils.

**Methods:** A flavonoid (PGA) and an alkaloidal compound (PGF) from the extract of *P. glabra* and a steroidal compound (FGS) and tannin fraction (FGT) from the extract of *F. glomerata* were isolated using column chromatography technique and were subjected for the spectroscopic (FT-IR, 1HNMR and LC-MS) and TLC studies to identify the compounds. The isolated compounds were screened for *in vitro* immunomodulatory activity on human neutrophils using nitroblue tetrazolium (NBT) dye test, phagocytosis of *Candida albicans* and neutrophil locomotion and chemotaxis assay at the concentration range of 100, 50, 25, 12.50 and 5.00 μg/mL.

**Results:** From the spectroscopic and TLC studies data, the isolated compounds were identified as glabrin (PGA), karanjin (PGF), β-sitosterol (FGS), and tannin fraction (FGT). The isolated compounds PGA, PGF, FGS, and FGT exhibited significant (*P* < 0.05) *in vitro* immunomodulatory activity in all the parameters studied.

**Conclusion:** The steroidal compound, i.e. FGS was found to be more immunopotent than all constituents alkaloid, flavonoid and tannins. Hence, these constituents could be attributed to the immunomodulatory property of the plants.

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1. **Introduction**

Immunotherapy is a part of modern system of medicine, an alternative approach for chemotherapy in dealing with the treatment of dreadful diseases like cancer, AIDS, etc. In Ayurveda, the term “Rasayana” is concerned with the chemical agents that modulate the immune response to alleviate the disease and thus the immunotherapy (Rasayana therapy) is not only beneficial in maintenance of health but also helpful in therapeutic management of disease state when used as an adjuvant or as a drug (Datt & Debnath, 1999).

The immunomodulatory property exhibited by plant products can be attributed to the phytoconstituents present in them, which act mainly on macrophages for generation of immune response (Dahanukar et al., 2000). The polysaccharides of *Chlorophytum borivilianum* (Safed musli, Liliaceae) showed significant stimulation of NK (natural killer) cells (Thakur et al., 2011). Flavonoids (proanthocyanidins) of *Pinus radiata* (Pinaceae) and *Tecoma undulata* (Bignoniaceae) were found to enhance the humoral and cell-mediated immune response in cyclophosphamide induced myelosuppressed mice (Choudhary, 2011). Ginsenosides (steroidal glycosides) of *Panax ginseng* (Araliaceae) have been shown to enhance NK cells, stimulate T-cell proliferation, augment phagocytosis, chemotaxis and production of IL-1, IL-2 and TNF-α (Kim et al., 1990). Alkaloids of *Solanum xanthocarpum* (Solanaceae) showed pronounced immune-protective activity by increasing the depleted levels of hematocytes (Sultana et al., 2011). Phenolic compounds (Tannins) of root extracts of *Ficus bengalensis* (Moraceae) showed significant immunomodulatory property (Singh et al., 2011).

*Pongamia glabra* Vent. and *Ficus glomerata* Roxb. are medium sized evergreen trees, which spread all over India. The plants and their classical preparations such as Karanja taila (*P. glabra*...
medicated oil), Mustakakaranjadi kvata churna (P. glabra and multi herbal medicated powder) and Udumbara Avalake (F. glomerata and multi herbal tonic) (The Ayurvedic Pharmacopoeia of India, 1999) are well known medicinal agents in Ayurvedic system of medicine. Traditionally, different parts including leaves, flowers, seeds and bark of P. glabra are used in different ailments such as bronchitis, whooping cough, rheumatic joints, anti-parasitic, flatulence and liver diseases (Kirtikar & Basu, 1995) and different parts of F. glomerata including leaves, flowers, fruits and bark are used in gynecological and urological disorders, hiccup, dysentery, diabetes, asthma and piles (Chopra et al., 1986).

Based on results of our earlier research works, which focus on in vivo and in vitro screening of immunomodulatory activity of extracts of seeds and stem bark of P. glabra (Sanjeev et al., 2012; Sanjeev et al., 2013a) and extracts of fruits and stem bark of F. glomerata (Sanjeev et al., 2013b), wherein the extracts of bark of both plants exhibited more significant (P < 0.05) immunomodulatory activity than the other plant part extracts. Hence the present study was designed to isolate vital phytoconstituents from stem bark extracts of the two plants and to characterize spectroscopically using FT-IR, 1HNMR and LC-MS techniques. The constituents were subjected for identification by interpreting the results with the reference compounds and screening for immunomodulatory activity using in vitro models – Nitroblue tetrazolium (NBT) assay, phagocytosis of Candida albicans and chemotaxis assay.

2. Materials and methods

2.1. Extraction of plant material

P. glabra and F. glomerata stem bark were collected from local areas of North Karnataka, India (GUG/BOT/Herbarium/2008–09/0 9). The plant materials were dried under shade and pulverized to particle size 40. The coarse powder was first subjected for defatting with petroleum ether (40–60 °C) and then extracted with methanol using Soxhlet apparatus at 40 °C for 48 h to obtain methanolic extracts of the plants. The filtrated extracts were concentrated to dryness at 40 °C using a rota flash evaporator (Pullock, 2002).

2.2. Phytochemical studies

Preliminary qualitative chemical analysis and thin layer chromatography (TLC) studies of methanolic extracts of the plants revealed the presence of flavonoids, alkaloids and sterols in the extract of P. glabra and sterols, saponins, tannins and glycosides in the extract of F. glomerata respectively. The major phytoconstituents of the extracts, i.e. alkaloid and flavonoidal compounds from the bark extract of P. glabra and steroidal compound and tannin fraction from the bark extract of F. glomerata were isolated using the following methods.

2.2.1. Isolation of alkaloid (PGA) from stem bark extract of P. glabra

Stas-Otto method was followed to separate the alkaloid from the plant. About 50 g of defatted methanolic extract of the plant was dissolved in sufficient quantity of distilled water and was acidified using 2 × 100 mL of dil. HCl to remove acid soluble non-alkaloidal impurities and then it was made alkaline using 2 × 100 mL of NH4OH and was filtered. The alkaline filtrate was further fractionated with 4 × 100 mL of chloroform. The organic fractions were pooled discarding the aqueous layer (acidic layer). The pooled organic fraction was evaporated to dryness to yield a gummy residue. The alkaloidal composition in the residue was confirmed using TLC method and the residue was further subjected to column chromatography using the mobile phase – toluene: ethyl acetate: diethyl amine (7:2:1). Eluates were collected and monitored by TLC studies. The eluates which gave single spot for alkaloidal compound in TLC studies were pooled, evaporated to dryness and purified using methanol (Hildebert & Sabine, 2001).

2.2.2. Isolation of flavonoid (PGF) from stem bark extract of P. glabra

About 50 g of defatted methanolic extract of the plant was dissolved in 10 mL of distilled water and 100 mL of ethyl acetate and shaken for several times in a separating funnel. The ethyl acetate fraction was separated and concentrated to dryness and subjected to column chromatography using the mobile phase – ethyl acetate: formic acid: glacial acetic acid: water (10: 1: 1: 2.6). Eluates were collected and monitored by TLC studies. The eluates which gave single spot for flavonoidal compound in TLC studies were pooled, evaporated to dryness and purified using methanol (Hildebert & Sabine, 2001).

2.2.3. Isolation of sterol (FGS) from stem bark extract of F. glomerata

About 70 g of methanolic extract was dissolved in water and filtered. The filtrate was extracted with n-hexane. The excess of solvent was removed by distillation and the n-hexane fraction was collected and concentrated to dryness using a rota flash evaporator. About 15 g of n-hexane fraction was subjected for saponification, which was followed by determination of unsaponifiable matter (Indian Pharmacopoeia, 1996).

An accurately weighed quantity of about 12 g of n-hexane fraction was taken in 250 mL RB (round bottom) flask fitted with reflux condenser. A 4% solution of potassium hydroxide in alcohol was added to this and heated on water bath for 1 h, shaking frequently. The content of the flask was then transferred to a separating funnel and fractionated 3–4 times with 100 mL of solvent ether. Ether extracts were combined and subjected for distillation to remove the solvent. About 8 g of a yellow residue of unsaponifiable matter was separated, which was confirmed for the content of sterols by TLC studies and the same was further subjected for column chromatography using petroleum ether: acetone (7:3) as mobile phase. The eluates were collected and monitored by TLC studies. The eluates which gave single spot for sterol compound in TLC studies were pooled, evaporated to dryness and purified by using solvent ether.

2.2.4. Isolation of tannin fraction (FGT) from stem bark extract of F. glomerata

About 20 g of methanolic extract of the plant was boiled with water for 30 min and cooled. The extract then was added with saturated solution of lead acetate to precipitate tannins and filtered. The filtrate was discarded and the residue collected was resuspended in dilute sulphuric acid solution to remove lead as lead sulphide. Then it was again filtered and the filtrate was concentrated to get crude tannins. Thus, the obtained concentrated product of crude tannins was purified by loading on Sephadex LH-20 column using the eluents – 50% methanol followed by 70% acetone. The eluted acetone fraction was concentrated using rota flash evaporator to yield the resulted product (Ronald et al., 2008).

2.3. Characterization of isolated compounds and fractions

The resulted compounds, viz. – PGA (alkaloid), PGF (flavonoid), FGS (steroid) and FGT (tannin fraction) were characterized by TLC studies and FT-IR, 1HNMR and LC-MS spectroscopic analysis.

2.4. In vitro screening of isolated compounds and fractions – PGA, PGF, FGS and FGT

2.4.1. Preparation of stock solutions

Stock solutions for in-vitro studies were prepared in the concentration range of 100, 50, 25, 12.50 and 5.00 μg/mL by dissolving the
isolated compounds and the fractions – PGA, PGF, FGS and FGT of stem bark extracts of *P. glabra* and *F. glomerata* respectively in 0.5 mL dimethyl sulphoxide (DMSO) and phosphate buffer salt solution (PBS solution – prepared by dissolving 2.38 g of disodium hydrogen phosphate, 0.19 g of potassium dihydrogen phosphate and 8.0 g of sodium chloride in sufficient water to produce 1000 mL).

### 2.4.2. Nitroblue tetrazolium (NBT) dye assay

A fresh solution of 0.3% of NBT was prepared in 0.34% of sucrose solution. One part of NBT solution was added to one part of PBS (phosphate buffer solution). Meanwhile a suspension of leucocytes (5 \( \times \) 10^5/ml) was also prepared in 0.5 mL PBS.

The stock solutions of the isolated compounds and the fraction – PGA, PGF, FGS and FGT in the concentration range of 100, 50, 25, 12.50 and 5.00 \( \mu \)g/ml were individually added with 0.2 mL freshly prepared 0.15% NBT solution and suspension of leucocytes.

In another test tube containing 0.1 mL of endotoxin activated plasma was added with the above solutions instead of test solutions and it was used as positive control (standard).

A normal control with only suspension of leucocytes and NBT solution in another test tube was also maintained. All the above solution containing test tubes were incubated at 37 °C for 20 min and centrifuged gently at 400g for 3–4 min. The supernatant obtained after centrifugation was discarded and a drop of PBS was added and gently resuspended the cells in small volumes of fluid at the bottom of the test tube. A drop of this fluid was allowed dry on a microscope slide and made into a film, which was fixed gently by heating and counterstained with dilute carbol-fuchsin.

Then it was washed, dried and mounted using a 100 \( \times \) oil immersion objective. About 200 neutrophils were counted and the percentage of NBT positive cells (contain blue deposits) was determined.

### 2.4.3. Phagocytosis of killed fungus (*Candida albicans*)

Saboraud 2% dextrose broth medium was selected for growing the *Candida albicans* by incubating the medium for 48 h at 37 °C. The cultures were spun at 1500 g for 10 min and using PBS the deposit was washed and filtered twice through sterile gauze. A concentration 5 \( \times \) 10^6 cell/ml *Candida albicans* was resuspended in Hank's (buffer) solution. The organisms were killed by heating at 100 °C for 30 min. The sterility checking for the heated batch was carried out and the culture was cooled and stored at 20 °C.

The solutions (0.25 mL) of various concentrations i.e., 100, 50, 25, 12.50 and 5.00 \( \mu \)g/ml of the isolated compounds and the fraction – PGA, PGF, FGS and FGT of stem bark extracts of *P. glabra* and *F. glomerata* were individually added with 0.25 mL Hank’s solution, 0.25 mL heat killed *Candida albicans* and 0.25 mL neutrophil suspension in separate test tubes.

In another test tube a positive control was maintained, which consisted of 0.25 mL of pooled serum added with the above solutions except the test extracts.

A normal control was also maintained in another test tube, which contained of 0.25 mL of neutrophil suspension, 0.25 mL of Hank’s solution and 0.25 mL of heat killed *Candida albicans*.

All the test tubes were subjected for slightly shaking for proper mixing of solutions. They were incubated at 37 °C for 30 min, centrifuged at 200 g for 5 min and using a pasteur pipette the supernatant removed leaving a droplet into which the sediment was resuspended. Smears were made, dried in air and stained with May-Grunwald Giemsa stain. About 200 neutrophils were examined, the number of ingested *Candida albicans* associated with each cell (neutrophil) was counted and the mean particle number associated with each neutrophil was calculated.

### 2.4.4. Neutrophil locomotion and chemotaxis assay

Chemotaxis assay can be carried out using a special self-constructed apparatus called chemotaxis assembly, which consists of an upper chamber containing the neutrophil cells suspension separated by a micropore filter from a lower chamber – which contains the chemotactic factor i.e. isolated compounds. The assembly is a self constructed apparatus using a 5 mL beaker in a sandwich box with holes bored in its lid contains the lower chamber and Sawnoff tuberculin syringe containing the upper chamber with the filter glued to its lower end is inserted through the hole into the lower chamber in the beaker.

About 10^6 cells/ml neutrophil cells were prepared in Hanks (buffer) solution. The lower chambers of the chemotaxis assembly with appropriate chemotactic agent maintained at a pH 7.2.

Chamber 1: Hank’s solution (Normal control)

Chamber 2: Casein 1 mg/ml (positive control)

Chamber 3: Pre-determined concentrations of the isolated compounds and the fractions – PGA, PGF, FGS and FGT (100, 50, 25, 12.50 and 5.00 \( \mu \)g/ml in separate chemotaxis assemblies).

The upper compartments filled with the neutrophil cells suspension ensuring that the fluid level in the upper and lower chambers was maintained the same to avoid gradient disturbance. The filters were allowed for wetting from the top before putting them into the lower compartments, when the contents of upper compartments were placed in the lower compartments. The concentration of chemotactic factor throughout the filter was zero before its placement in the lower compartment and after placement of the filtered in chemotactic solution, the gradient began to form the bottom of the filter. The arrangement was maintained steady until the end of the experiment. Then it was incubated at 37 °C in air for 3 h. After about 45 min the upper compartments were removed from the assembly and inverted them to empty fluid out of them.

The fluid was fixed by immersing the filters in 70% ethanol or methanol. After few minutes immersion in alcohol, the glue melted and the fluid became loose, which was picked off gently using dental packing forceps. It was stained with haematoxylin and xylol respectively, mounted under microscope using glycerin and covered with a coverslip. The cell migration was measured microscopically by counting the number of cells that have reached the lower surface of the filter after a given time interval. The count of lower surface was taken, as the count of cells on the lower surface is directly proportional to the number of cells placed on top of the filter at the start of the experiment (Wilkinson, 1981; Reevas, 1994; Chaining & Rodger, 1994).

### 2.5. Statistical analysis

Data were expressed as mean ± SEM and differences between the groups were statistically determined by analysis of variance (ANOVA) followed by Dunnet’s test.

### 3. Results

#### 3.1. Isolation and characterization of phytoconstituents

*P. glabra* (alkaloidal compound) was obtained as a pale greenish brown solid mass (yield – 481 mg, MP 291 °C), which was confirmed to be an alkaloidal compound by preliminary qualitative chemical tests and TLC study (Wagner’s reagent spray, Brownish spot; Rf = 0.51). FT-IR, ^1^HNMR and LC-MS spectral studies of the compound, PGA revealed the presence of N=CH group, OH groups and the COOH groups. The ^1^HNMR spectrum accounted for 13 protons and the LC-MS gave a molecular ion peak at m/z 176 corresponding to the molecular weight of the compound (Table 1, Fig. 1A).
chemical tests and TLC studies (blue fluorescent spot in UV 365, Rf confirmed to be a flavonoidal compound by preliminary qualitative lowish brown solid mass (yield – 184 mg, MP 157°C) to FGS.

white solid mass (yield – 258 mg, MP 131°C) & slight heating in oven at 105°C, Dark purple colored spot, Rf confirmed to be a steroidal compound by preliminary qualitative chemical tests and TLC studies (Anisaldehyde-Sulphuric acid spray dark brown to black solid mass (yield – 436 mg), which was confirmed to be a alkaloidal compound).

PGF (P. glabra Flavonoid compound) was obtained as a pale yellowish brown solid mass (yield – 184 mg, MP 157°C), which was confirmed to be a flavonoidal compound by preliminary qualitative chemical tests and TLC studies (blue fluorescent spot in UV 365, Rf value – 0.71). The spectral studies of the compound, PGF revealed the presence of aromatic C-H groups, -OCH3 group, C=C and C=O groups and monosubstituted phenyl ring. The 1H NMR spectrum accounted for 12 protons and the LC-MS gave a molecular ion peak at m/z 293 corresponding to the molecular weight of the compound (Table 2, Fig. 1B).

FGS (F. glomerata Steroidal compound) was obtained as a pearl white solid mass (yield – 258 mg, MP 131°C), which was confirmed to be a steroidal compound by preliminary qualitative chemical tests and TLC studies (Anisaldehyde-Sulphuric acid spray & slight heating in oven at 105°C. Dark purple colored spot, Rf value – 0.42). The spectral studies of the compound, FGS showed the presence of OH, C=C, CH2 and CH3 groups. The 1H NMR spectrum accounted for 50 protons and the LC-MS gave a molecular ion peak at m/z 416 corresponding to the molecular weight of the compound (Table 3, Fig. 1C).

FGT (F. glomerata Tannin fraction) was obtained as a brownish black solid mass (yield – 436 mg), which was confirmed to be a tannin fraction by preliminary qualitative chemical tests and TLC studies (Light violet colored spot, Rf value – 0.65).

3.2. Nitroblue tetrazolium (NBT) dye assay

The isolated compounds – PGA, PGF, FGS and FGT were found to produce the phagocytosis to the extent of 46.60 ± 9.537, 26.80 ± 1.393, 44.20 ± 2.80 and 38.80 ± 3.121 percentage respectively at the above mentioned concentration range. They exhibited significant (P < 0.001) activity in comparison with the positive control i.e. endotoxin activated plasma (86%). However the PGA was found to produce higher percentage of phagocytic activity than the PGF and similarly the FGS was found to produce higher percentage of phagocytic activity than the FGT and the activity shown by them was concentration dependent (Table 4).

3.3. Phagocytosis of killed fungus (Candida albicans)

The mean particle numbers (MPN) of phagocytosed Candida albicans for the isolated compounds – PGA, PGF, FGS and FGT were found to be 3.00 ± 0.447, 2.80 ± 0.374, 3.20 ± 0.374 and 3.60 ± 0.678 respectively at the above mentioned concentrations. PGA, PGF, FGS and FGT exhibited significant (P<0.05, P<0.01) phagocytic activity

Table 1

| IR results of PGA | Functional groups | 1H NMR results of PGA | LC-MS results (m/z) |
|------------------|-------------------|----------------------|---------------------|
| Wave No./cm⁻¹   |                   | Chemical shifts (δ × 10⁻⁵) | Type of proton       |
| 3425             | OH stretch of Ar. –OH & COOH | 1.2692 | 3 H, s, 3 H of N-CH3 & C4  |
| 2570 & 2844      | C=H stretch of CH2  | 2.2451 | 2 H, 2 H at ring C2 & C4  |
| 1698.63          | C=O of COOH       | 2.6745 | 5 H, 5 H of ring          |
| 1446 & 1364      | C=H bend of CH2   | 7.8127 | 1 H, s, OH at C4          |
| 1654             | C=N stretch       | 8.1835 | 1 H, s, OH at C3          |
| 1211 & 1112      | O=H of OH        |         |                      |
| 1035             | C=O              |         |                      |
| 931              | Substituted ring  |         |                      |

Table 2

| IR results of PGA | Functional groups | 1H NMR results of PGA | LC-MS results (m/z) |
|------------------|-------------------|----------------------|---------------------|
| Wave No./cm⁻¹   |                   | Chemical shifts (δ × 10⁻⁵) | Type of proton       |
| 3052             | Ar. C=H stretch   | 3.93 | 3 H, s, 3 H of –OCH3     |
| 2910 & 2849      | C=H stretch of –OCH3 | 7.2 to 7.4 | 9 H, m, Ar. heterocyclic ring |
| 1731             | Ring C=O         |         |                      |
| 1600             | C=C ring stretch |         |                      |
| 1441, 1364       | C=C bend of –OCH3 |         |                      |
| 1260             | C=O              |         |                      |
| 1112&1041        | C=O=C            |         |                      |
| 728              | Monosubstituted Phenyl ring |         |                      |

Table 3

| IR results of PGA | Functional groups | 1H NMR results of PGA | LC-MS results (m/z) |
|------------------|-------------------|----------------------|---------------------|
| Wave No./cm⁻¹   |                   | Chemical shifts (δ × 10⁻⁵) | Type of proton       |
| 3416.3           | OH stretch        | 5.1 | m, 1 H vinylic at C6   |
| 2853 & 2720      | C=H stretch of CH3 & CH2 | 3.5 | s, 1 H, CH=OH at C1   |
| 1635.6           | C=C stretch       | 2.3 to 1.4 | m, 8 H, of –CH2       |
| 1463 & 1376      | C=H bend of CH3 and CH2 | 1.3 to 1.2 | m, 22 H, 11 × CH1    |
| 1133.3           | OH bend           | 1.0 to 0.8 | m, 18 H, 6 × CH3     |

Fig. 1. A. Structures of glabrin (2-piperidine, carboxylic acid, 4,5-dihydroxy-1-methyl) – reference compound to PGA; B. Karanjin (3-methoxy-2-phenyl-4-(2,3-h)chromen-4-one) – reference compound to PGF; C. β-sitosterol – reference compound to FGS.
compared to the positive control i.e. pooled serum. However, the MPN for the isolated compound, PGA was higher than that of PGF and similarly MPN for the FGT was higher than that of FGS (Table 5).

3.4. Neutrophil locomotion and chemotaxis assay

The chemotactic activity of the isolated compounds – PGA, PGF, FGS and FGT were significant \( (P < 0.05, P < 0.01) \) and the average count of the mean number of neutrophils attracted per field for the isolated compounds, PGA, PGF, FGS and FGT were found to be 126.20 ± 23.12, 112.80 ± 12.92, 133.00 ± 13.91 and 88.40 ± 16.91 respectively in comparison with the positive control i.e. casein (200). However, the chemotactic activity of PGA was comparatively higher than that of PGF and similarly the activity of FGS was higher than that of FGT (Table 6).

4. Discussion

Immunostimulation is the choice of treatment during conventional chemotherapy when the impaired host immune responsiveness is required to be activated and to provide prophylaxis of infection in sensitive patients.

The spectral data of the isolated compound, PGA suggested its structural resemblance with the reference alkaloidal compound, glabrin (pipecolic acid derivative; alkaloidal compound; molecular formula: C7H13NO4; molecular weight: 175). Similarly the spectral details of PGF suggested its structural resemblance with the reference flavonoid compound, karanjin (furanoflavonoid compound; molecular formula: C18H12O4; molecular weight: 292). In this way an alkaloidal and a flavonoid compounds have been isolated from stem bark extract of \( P. \) glabra and they were identified as glabrin and karanjin respectively by referring to their spectral data (Fig. 1A and B).

| Table 5 |
|---|
| Phagocytosis assay of killed \( C. \) albicans for isolated compounds and fractions (PGA, PGF, FGS and FGT) from methanolic extracts of stem bark of \( P. \) glabra and \( F. \) glomerata on human neutrophils. |
| Concentrations | Mean particle number (MPN) |
| Normal control | Pooled serum (Positive control, 1 mg/mL) |
| PGA | PGF | FGS | FGT |
| 5 \( \mu \)g/mL | 2 ± 0.0 | 23 | 24 | 35 | 30 |
| 12.5 \( \mu \)g/mL | 32 | 23 | 41 | 35 |
| 25 \( \mu \)g/mL | 25 | 40 | 28 | 38 |
| 100 \( \mu \)g/mL | 70 | 30 | 51 | 48 |
| Mean ± SEM | 46.60 ± 9.54 | 26.80 ± 1.39 | 44.20 ± 2.80 | 38.80 ± 3.12 |

Note: Significant difference from positive control by One-way ANOVA followed by Dunnet’s ‘t’ test \( (n = 4) \).

Test extract treated groups were compared with positive control group.

Values of significance – a = \( P < 0.05 \), b = \( P < 0.01 \), ns- non-significant.

| Table 6 |
|---|
| Neutrophil locomotion and chemotaxis assay for isolated compounds and fractions (PGA, PGF, FGS and FGT) from methanolic extracts of stem bark of \( P. \) glabra and \( F. \) glomerata on human neutrophils. |
| Concentrations | Mean number of neutrophils per field |
| Normal control | Casein (Positive control, 1 mg/mL) |
| PGA | PGF | FGS | FGT |
| 5 \( \mu \)g/mL | 15 ± 0.0 | 55 | 88 | 111 | 55 |
| 12.5 \( \mu \)g/mL | 111 | 88 | 111 | 66 |
| 25 \( \mu \)g/mL | 111 | 100 | 111 | 66 |
| 50 \( \mu \)g/mL | 177 | 144 | 155 | 111 |
| 100 \( \mu \)g/mL | 177 | 144 | 177 | 144 |
| Mean ± SEM | 126.20 ± 23.12 | 112.80 ± 12.92 | 133.00 ± 13.91 | 88.40 ± 16.91 |

Note: Significant difference from positive control by One-way ANOVA followed by Dunnet’s ‘t’ test \( (n = 4) \).

Test extract treated groups were compared with positive control group.

Values of significance – a = \( P < 0.05 \), b = \( P < 0.01 \), c = \( P < 0.001 \).
The spectral data of the isolated compound, FGS suggested its structural resemblance with the reference steroidal compound, β-sitosterol (Steroidal compound – Molecular formula – C_{29}H_{50}O, Molecular weight – 414). In this way a steroidal compound has been isolated from stem bark extract of *F. glomerata* and it was identified as β-sitosterol by referring to its spectral data (Fig. 1C). A tannin fraction, FGT was also isolated from stem bark extract of the same plant. The tannin fraction was a complex compound; its spectral analysis could not be possible and hence it was confirmed by TLC studies and preliminary qualitative analysis.

Nitroblue tetrazolium (NBT) assay is a qualitative test, in which stimulated (opsonized) neutrophils can be seen as formazone granules that are formed by absorbing nitroblue tetrazolium stain. Mean percentage of reduced neutrophils was noted with the isolated compounds (PGA, PGF, FGS and FGT) against positive control i.e., endotoxin activated plasma.

Phagocytosis of killed fungus (*Candida albicans*) assay is a method of assessing phagocytosis, which relies on the ingestion of particles by phagocytes over a brief period. In this the number of foreign particles phagocytosed by neutrophil cells was counted under the microscope.

Neutrophil locomotion and chemotaxis assay is a process of chemotaxation i.e., attraction of neutrophils towards certain chemicals called chemoattractants. This is a micropore filter method, in which the isolated compounds (PGA, PGF, FGS and FGT) are tested to contain chemoattractants and the mean number of neutrophils attracted per field was noted against positive control – casein.

The results of the study parameters, viz. – NBT assay, phagocytosis and chemotaxis assay indicate opsonization of neutrophils, particle ingestion and chemoattraction of neutrophils respectively, which collectively contribute to the process of immunomodulation (immunostimulation) of the isolated compounds and the fraction – PGA, PGF, FGS and FGT of stem bark extracts of *P. glabra* and *F. glomerata* at various concentration range.

The spectral data and interpretation with the reference compounds suggest the isolated compounds – PGA and PGF to be identified as glabrin (*Subbarao & Veerabhadra Rao*, 1941) (alkaloidal compound), karanjin (*Seshadri & Venkateswarlu*, 1941) (flavonoidal compound) respectively, of stem bark of the plant, *P. glabra* and FGS as β-sitosterol (*Baslas and Agha*, 1985) (steroidal compound) respectively, of stem bark of *F. glomerata*. A tannin fraction, FGT was also isolated from the latter plant extract and confirmed its identity by TLC and preliminary phytochemical studies.

In vitro immunomodulatory activity screening of the isolated compounds and the fraction showed FGS (steroidal compound) and PGA (alkaloidal compound) to have significant activity than the other two. Comparatively the steroidal compound exhibited more significant phagocytic and chemotactic effects. Steroidal glycosides are vital phytococonstituents, which have been proven to have different pharmacological activities such as cytotoxic, antitumour, antiviral, antiallergic, antipathotoxic, antifungal and immunomodulating activities (*Zhang et al.*, 2007). For e.g.: steroidal glycosides, viz – gensenosides from *Panax ginseng*, kutskoses from *Picrorrhiza kurroa*, etc. have been reported for immunostimulating activity.

5. Conclusion

It can be concluded from the study that the alkaloid and flavonoidal compounds of *P. glabra* and the steroidal compound and tannins of *F. glomerata* may be attributed to the immunomodulatory property of the plants.

**Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

**Acknowledgment**

Authors are thankful to authorities of HKE Society and MTR Institute of Pharmaceutical sciences, Kalaburagi, Karnataka India, for providing necessary facilities to carry out the study.

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