Phytochemical Composition and Evaluation of Anti-Inflammatory Activity in *Glycosmis pentaphylla* (Retz.)DC. - An Ethnobotanically Important Medicinal Plant

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Abstract The present study was undertaken with an objective to investigate the phytochemical composition and *in vitro* anti-inflammatory potential in the leaves of *Glycosmis pentaphylla* (Retz.) DC, an ethno-medicinally important medicinal plant belonging to the Rutaceae family. Determination of secondary metabolites in any plant material is necessary as it helps to know about the major constituents present in them. Extractants like hexane, ethyl acetate, acetone, ethanol and distilled water were selected based on the polarity of the solvents used and was further evaluated for phytochemicals through qualitative and quantitative analysis based on standard procedures. Inflammation is a part of body's immune response and it is necessary to heal the tissue damage through an inflammatory response. As modern non-steroidal anti-inflammatory drugs can cause severe side effects like depression, gastric problems and so on, it is advisable to use plant derived products to treat inflammation. In this context, the potential of *Glycosmis pentaphylla* as an anti-inflammatory agent has been critically evaluated using *in vitro* cyclooxygenase and lipoxygenase inhibitory assays. The results revealed that major secondary metabolites evaluated were present in the ethanolic extract, indicating that ethanol can dissolve most of the phytoconstituents. Also, on quantification, it was found that the leaf ethanolic extract possessed alkaloids as a major constituent. As secondary metabolites can possess several pharmacological activities like anti-bacterial, anti-fungal, anti-tumour and so on, the potential of *Glycosmis pentaphylla* in elucidating anti-inflammatory effects was checked through *in vitro* anti-inflammatory assays. It was observed that, the leaf extract showed promising cyclooxygenase and lipoxygenase inhibition indicating that,

the extract can be used as an effective non-steroidal anti-inflammatory drug. From the phytochemical composition, it can be gleaned that, the anti-inflammatory activity might be due to the presence of alkaloids. Hence, this research work can aid the pharmaceuticals to isolate herbal anti-inflammatory drugs rich in alkaloids from *Glycosmis pentaphylla*.

Keywords *Glycosmis pentaphylla*, Phytochemicals, Anti-Inflammatory Activity, Lipoxygenase, Cyclooxygenase

1. Introduction

Inflammation may be a complex process, related to pain and involves disturbances like increase in vascular permeability, protein denaturation and membrane alteration. As it is one of the body's nonspecific internal systems of defense, the response of a tissue to an accidental cut is similar to the response that results from other types of tissue damage. The establishment of new *in vitro* test systems has stimulated the screening of plants aiming to find leads for the development of new drugs. Non-steroidal anti-inflammatory drugs (NSAIDs) represents the most common classes of medications used worldwide for inflammation and related disorders [1]. NSAIDs available in the market are carboxylic acid containing drugs including salicylate derivatives (2-acetoxybenzoic acid), carboxylic and heterocyclic acid derivatives (1-(p-chlorobenzoyl) 25 methoxy-2- methylindole-3-acetic acid), propionic acid derivatives [2-(4-isobutylphenyl)

propionic acid], 2-(3-Benzophenyl) propionic acid) and phenyl acetic acid derivatives [2-(2,6-dichloroanilino) phenyl) acetic acid]. These organic acid containing drugs act at the active site of the enzyme preventing the access of arachidonic acid (AA) to the enzyme and stop the cyclooxygenase pathway [2,3]. In spite of the excellent anti-inflammatory potential of the NSAIDs, the severe side effects such as gastrointestinal (GI) ulceration, perforation, obstruction, and bleeding has limited the therapeutic usage of NSAIDs. In this scenario, usage of an alternative drug can serve the cause. Hence, there comes the role of herbal medicines. In spite of their incredible health benefits, overmedication might lead to health hazards. Researchers are therefore developing standard drug formulations from medicinal plants, which can be of potential use. This study is thus significant in standardizing a potent anti-inflammatory agent from *Glycosmis pentaphylla*.

The significance of using medicinal plants can be attributed to a number of reasons, including affordability and limited availability of Western medicine to the underdeveloped nations as well as the trust in herbal medicine as an outcome of the witnessed positive results when applying herbs [4]. One of the many reasons that prompted indigenous people from such communities to rely heavily on plant-based traditional herbs is of the high cost of conventional medicines [5]. Therefore, plants are of infinite value to less affluent populations [6].

Glycosmis pentaphylla, commonly called as orange berry belonging to the family Rutaceae is a shrub or small (1.5–5 m) tree. It is distributed, spanning from India, Malaysia and Southern China to the Philippine Islands where it occurs in tropical forests at low altitudes. Traditionally, it is known to be useful for the treatment of wide panel of diseases like cough, rheumatism, anaemia and jaundice. Some of the major classes of compounds reported from *G. pentaphylla* include terpenoids, amides, imides, alkaloids, coumarin, and flavonoids [7].

As plant phytoconstituents can produce a pharmacological effect, in the present work, an attempt to correlate the efficacy of secondary metabolites in elucidating pharmacological responses is also analysed. Even though, several investigations on the plant for hepatoprotective activity [8] and antipyretic potential [9] have been done, there are only few reports on the anti-inflammatory activity of *G. pentaphylla*. Hence, this work can provide novel information for peer groups to determine and quantify the phytoconstituents and standardize the leaf extract as an anti-inflammatory agent using cyclooxygenase and lipoxygenase guided activity.

2. Materials and Methods

2.1. Collection and Authentication of Plant Material

Fresh young sunny leaves of *Glycosmis pentaphylla*

harvested about ten weeks after bottom pruning and subsequent picking at an interval of 7 to 8 weeks in triplicates were collected during spring season from the district of Thiruvananthapuram (Latitude- 8.54°N, Longitude- 76.91°E and Altitude 18.00 m), Kerala, India. The botanical identities were done by Curator, Department of Botany, University of Kerala, Thiruvananthapuram, Kerala, India and a voucher specimen was deposited at the Department Herbarium with an accession code KUBH 6043. The leaves were washed under running tap water, rinsed with distilled water, dried under shade at room temperature for two weeks. The dried leaves were powdered using electric blender, sift through 0.420 mm mesh sieve and stored in airtight bottles to free from moisture and humidity until further experimental usage.

2.2. Preparation of Plant Extract

About 10 mg of ground leaf powder was subjected to successive soxhlet extraction using the solvents n-hexane (0.10), ethyl acetate (4.4), acetone (5.1), ethanol (6.5) and distilled water (10.2) one after the other based on their polarity [10]. The polarity was determined depending on low boiling points as well as their ability to evaporate. The samples were extracted for 5 hours and the extractants were filtered and concentrated using a rotary evaporator to obtain solid masses. The filtrate was evaporated inside an oven at a temperature of 50°C.

2.3. Qualitative Analysis of Phytochemicals

Various chemical tests were performed for the presence of phytochemical constituents using standard procedures. The qualitative estimation of secondary metabolites was studied as per Harborne [11]. The tests for various classes of secondary metabolites were as follows:

2.3.1. Tannins

Presence of tannins was determined using the protocol reported by Sofowara [12]. Boiled 50 mg of each crude extract obtained by soxhlet extraction in distilled water and filtered. A few drops of 0.1% Ferric chloride (FeCl₃) was mixed and observed separately for colour change. The presence of brownish green colouration shows the occurrence of tannins.

2.3.2. Saponins

Determination of saponins was carried out according to the standard procedure of Harborne [11]. In this method, 20 mg of each crude extract was boiled, filtered and mixed with a few ml of olive oil. Formation of emulsion revealed the presence of saponin.

2.3.3. Flavonoids

Methods of Harborne [11] and Sofowara [12] were used for the identification of flavonoids. A few ml of 1 %

ammonia (NH_3) solution was added to 10 mg of the crude extracts separately in a test tube. Presence of flavonoids was detected by yellow colouration.

2.3.4. Terpenoids

Presence of terpenoids in the crude extracts were determined by Salkowski test [11]. Mixed 5 mL (1 mg/mL) of extract with few drops of chloroform, and then 3 mL of concentrated H_2SO_4 . An interface with a reddish brown colouration indicated the presence of terpenoids.

2.3.5. Alkaloids

Alkaloids in all the crude extracts were detected according to Harborne [11]. Mixed 10 mg of extracts separately with 8 mL dilute Hydrochloric acid (1% HCl). It was warmed, filtered and the filtrate was treated 2 mL Dragendroff's reagent. Orange colour of the precipitate indicated the presence of alkaloids.

2.3.6. Cardiac Glycosides

Presence of cardiac glycosides in each extracts were carried out according to the method of Trease and Evans [13]. Each of the crude extracts (10 mg) was mixed with 2 mL of glacial acetic acid. The mixture was carefully added to 1 mL of concentrated H_2SO_4 taken in a test tube. If cardiac glycoside is present in the sample, a brown ring will appear between the upper organic layer and lower sulphuric acid layer.

2.3.7. Coumarins

Presence of coumarins was determined according to Trease and Evans [13]. Took 10 mg of each extracts separately in a test tube and covered it with filter paper moistened with 1 N Sodium hydroxide (NaOH). The test tube was placed in boiling water bath for a few minutes. After removing the filter paper, it was examined under UV light. Yellow fluorescence indicated the presence of coumarins.

2.3.8. Anthraquinones

Presence of anthraquinones was determined according to Trease and Evans [13]. Boiled 200 mg of crude extracts separately with 6 mL of 1 % Hydrochloric acid (HCl) and filtered. The filtrate was shaken with 5 mL of benzene. The aqueous layer was removed and 10 % Ammonium hydroxide (NH_4OH) was added to the alkaline phase. Formation of pink, violet or red colour in the alkaline phase confirmed the presence of anthraquinones.

2.4. Quantitative Analysis of Phytochemicals

The quantitative estimation of phytoconstituents were carried out according to the standard procedures of

Harborne [11].

2.4.1. Alkaloids

The plant extracts (10 mg) were dissolved separately in 2 N Hydrochloric acid (HCl) and then filtered and washed with 10 mL chloroform. One mL of this solution was transferred to a separating funnel. The pH of this solution was adjusted to neutral with 0.1 N Sodium hydroxide (NaOH). Then, 5 mL of bromocresol green solution and 5 mL phosphate buffer were added to this solution. The mixture was shaken and the complex formed was extracted with 1, 2, 3 and 4 mL chloroform by vigorous shaking. The extracts were collected in a 10 mL volumetric flask and diluted to volume with chloroform. The absorbance of the complex in chloroform was measured at 470 nm. A standard curve was drawn using atropine (50, 75, 100, 125, 150, 175 and 200 mg/L) as standard. The percentage weight of total alkaloids with respect to the weight of plant material was calculated. All samples were run in triplicates.

2.4.2. Total Phenol

Added 200 μL (1-5 mg/mL) of each crude extracts solution to 1 mL Folin-ciocalteu reagent and was mixed and incubated. To this mixture 7 mL of 0.115 mg/mL Sodium carbonate (Na_2CO_3) was added. The resulting solution was incubated further for 2 hours and absorbance readings were taken at 765 nm using UV-Vis Spectrophotometer (Shimadzu UV PC-1600). A standard curve was drawn using gallic acid (50, 75, 100, 125, 150, 175, 200 mg/L) as standard. The percentage weight of total phenolics with respect to the weight of plant material was calculated. All samples were run in triplicates.

2.4.3. Total Flavonoid

Mixed 0.25 mL of each crude extracts (1-5 mg/mL in respective solvent) separately with 1.25 mL of deionized H_2O and then added 75 μL of 5 % (w/v) solution of Sodium nitrate (NaNO_3). After 6 minutes of incubation, 150 mg Aluminium chloride (10 % AlCl_3) solution was mixed and incubated for 5 minutes with addition of 1 molar Sodium hydroxide solution (NaOH). Optical density was deliberated instantly using UV-Vis Spectrophotometer (Shimadzu UV PC-1600) at 510 nm. A standard curve was drawn using quercetin (50, 75, 100, 125, 150, 175, 200 mg/L) as standard. The percentage weight of total flavonoid with respect to the weight of plant material was calculated. All samples were run in triplicates.

2.5. *In vitro* Anti-Inflammatory Assay

Anti-inflammatory assay was conducted for cyclooxygenase and lipoxygenase as described below;

2.5.1. Cyclooxygenase (COX) Assay

RAW 264.7 cell line was purchased from NCCS, Pune and was maintained in Dulbecco's modified eagles media

(Himedia, India) supplemented with 10% fetal bovine serum (Himedia, India) and grown to confluence at 37°C at 5% CO₂ in a CO₂ incubator. The cyclooxygenase activity was assayed by the method of Gierse [14] with slight modifications. The cell lysate in Tris-HCl buffer (pH 8) was incubated with glutathione 5mM/L, and haemoglobin 20 µg/L for one minute at 25°C. To this mixture, 10 mg of the ethanolic crude extract was added. The reaction was initiated by the addition of arachidonic acid 200 mM/L and was terminated after 20 minutes of incubation at 37°C, by the addition of 10% trichloroacetic acid in 1 N hydrochloric acid. After the centrifugal separation and the addition of 1% thiobarbiturate, cyclooxygenase activity was determined by reading absorbance at 632 nm using UV-Vis Spectrophotometer (Shimadzu UV PC-1600).

Percentage inhibition of the enzyme was calculated as,

$$\% \text{ inhibition} = \frac{[\text{Absorbance of control} - \text{Absorbance of test}]}{\text{Absorbance of control}} \times 100$$

2.5.2. Lipooxygenase (LOX) Assay

The determination of lipooxygenase activity was done as per Hirata [15]. The reaction mixture (2 mL final volume) contained Tris-HCl buffer (pH 7.4), 50 µL of cell lysate, and sodium linoleate (200 µL; 10mg/ml). The LOX activity was monitored as difference in absorbance at 234 nm, as linoleic acid shows absorbance at this wavelength and it reflects the formation of 5-hydroxyeicosatetraenoic acid from linoleate. Percentage inhibition of the enzyme was calculated using the formula prescribed for cyclooxygenase assay.

2.6. Calculations of 50% Inhibitory Concentrations (IC₅₀)

The half maximal inhibitory concentration (IC₅₀) is a measure of the effectiveness of a compound in inhibiting biological or biochemical function. This quantitative measure indicates how much of a particular drug or other substance is needed to inhibit a given biological process by half. In other words, it is the half maximal (50%) inhibitory concentration (IC) of a substance (50 % IC, or IC₅₀). Sometimes, it is also converted to the p IC₅₀ scale (-log IC₅₀), in which higher values indicate exponentially greater potency. According to the Food and Drug Administration (FDA), IC₅₀ represents the concentration of a drug that is required for 50% inhibition *in vitro*. The concentration of the crude extract that was required to scavenge 50% of the enzyme inhibitory activity was calculated by using the percentage inhibitory activities versus the concentration of crude extract using nonlinear regression analysis (curve fit) at five different concentrations of the extract and standard, as well.

3. Results

Plants owe their therapeutic potential by the presence of secondary metabolites. The present study carried out on leaves of *Glycosmis pentaphylla* revealed the presence of medicinally active constituents. The results of preliminary phytochemical screening show that alkaloids were present in all the solvent systems. Flavonoids and cardiac glycosides were present in all the solvent system except in distilled water (Table 1).

Table 1. Phytochemical composition of the leaves of *Glycosmis pentaphylla*

Secondary metabolites	Solvents				
	I	II	III	IV	V
Tannin	+	+	-	+	+
Saponin	-	-	+	+	-
Flavonoids	+	+	+	+	-
Terpenoids	+	-	+	+	+
Alkaloids	+	+	+	+	+
Cardiac Glycosides	+	+	+	+	-
Coumarins	+	+	-	+	+
Anthraquinones	-	+	-	-	-

I-Hexane, II-Ethyl Acetate, III-Acetone, IV- Ethanol, V-Distilled Water

‘+’ indicates presence and ‘-’ indicates absence

Quantitative estimation of the chemical constituents was conducted only for selected phytoconstituents based on the literature review. Hence, spectrophotometric assays were conducted only for alkaloids, phenols and tannins as the plant was found to be rich in these constituents. The results showed that the leaf ethanolic extract of *Glycosmis pentaphylla* contained higher levels of total alkaloid, phenol and flavonoid. Among the metabolites quantified, ethanolic extract showed copious amount, of which alkaloids was found to be in abundant. From the results, it was seen that, the flavonoid contents of the extracts were found to be in the following order: ethanol > n-hexane > ethyl acetate > acetone > distilled water. At the same time, the phenol contents of the extracts were found to be in the following order: ethanol > ethyl acetate > acetone > n-hexane > distilled water (Table 2). The total alkaloid content was found in the following order: ethanol > acetone > n-hexane > ethyl acetate > distilled water.

Table 2. Quantitative estimation of phytoconstituents in *G. pentaphylla* leaves

Parameters	Solvents	Leaf
Total flavonoid (mg/g)	n-Hexane	13.41±0.34
	Ethyl acetate	12.99±0.39
	Acetone	11.9±0.60
	Ethanol	15.3±0.44
	Distilled Water	8.44±0.76
Total phenol (mg/g)	n-Hexane	17.50±0.33
	Ethyl acetate	47.96±0.53
	Acetone	27.50±0.83
	Ethanol	54.5±0.35
	Distilled Water	44.5±0.93
Total alkaloid (mg/g)	n-Hexane	50.13±0.85
	Ethyl acetate	44.3±0.43
	Acetone	54.83±1.74
	Ethanol	88.4±1.18
	Distilled Water	36.46±1.65

Data taken in triplicates as mean ± standard error

As the crude ethanolic extract possessed more number of phytoconstituents, this was subjected to *in vitro* anti-inflammatory assay. The crude extract of *G. pentaphylla* inhibited both cyclooxygenase and lipoxygenase enzymes effectively at increasing concentrations. It can be gleaned from the results that, there is a gradual increase in percentage inhibition as concentration increases for the crude extracts. The extract inhibited cyclooxygenase enzyme with a half maximal inhibitory concentration (IC₅₀) value of 78.68µg/mL while lipoxygenase enzyme was inhibited with a half maximal inhibitory concentration (IC₅₀) value of 87.15µg/mL. Table 3 and 4 depict the percentage inhibition of cyclooxygenase

and lipoxygenase enzymes along with standard anti-inflammatory drug 2-[2-(2, 6-dichloroanilino) phenyl] acetic acid (diclofenac).

Table 3. Result of Cyclooxygenase (COX) assay of leaf extract

Sl.No	Concentration (µg/mL)	Percentage Inhibition	
		Diclofenac	Ethanolic extract
1	6.25	3.07	4.61
2	12.5	14.5	16.92
3	25	19.92	24.61
4	50	36.07	46.15
5	100	49.28	55.38

Table 4. Lipoxygenase (LOX) assay of leaf extract

Sl.No	Concentration (µg/mL)	Percentage Inhibition	
		Diclofenac	Ethanolic extract
1	6.25	3.92	5.92
2	12.5	10.31	11.4
3	25	23.73	26.97
4	50	31.39	37.28
5	100	49.88	52.63

4. Discussion

In plants, the medicinal value of the secondary metabolites is due to the presence of chemical substances that produce a definite physiological action on the human body [16,17]. In pharmacological studies, it has been reported that, secondary metabolites find applications in the treatment of several ailments. For example, alkaloids were found to have antifungal, analgesic, anti-inflammatory and anti-tumour activities. Hence, a knowledge on determination of phytochemical composition in plants is very essential. In the present study, a preliminary phytochemical screening on both the qualitative and quantitative grounds of the crude leaf extracts in *Glycosmis pentaphylla* was done. Extractants were selected based on their polarity and the results revealed that majority of the phytoconstituents were present in the ethanolic extract. At the same time, the alkaloids were present in all the solvent system used for the study. The results were in accordance with the work conducted by Arora [18]. As preliminary qualitative assays cannot determine the quantity of the chemical constituents, quantification of the metabolites was done. From the results, it was found that, the metabolites analysed possessed abundance in the ethanolic extract. This might be due to the high polarity of ethanol [19]. As successive serial extraction was employed, the phytoconstituents got dissolved maximum in ethanol than the most polar distilled water. That might be the reason for low amount of

metabolites in distilled water. Hence, this data on preliminary phytochemical screening can easily guide researchers to use ethanolic extract for further research works.

In the second part of work, focus was given to anti-inflammatory activity studies as there are only few reports of this on *G. pentaphylla*. The presence of alkaloids might be responsible for the obvious anti-inflammatory activities of the extracts of the plant. This is in line with the plethora of literature [20]. A significant inhibitory potential has also been demonstrated by flavonoids against a wide array of enzymes such as phosphodiesterases, phospholipase A₂, protein tyrosine kinases, protein kinase C, and others [21]. At an equivalent time, phenols also perform its anti-inflammatory action by reducing the cells that expresses inducible nitric acid synthase (iNOS) or by inhibiting the production of nitric oxide by decreasing iNOS expression [22]. These data predict the high potential of phytochemical constituents to inhibit inflammation processes. In the present study, *in vitro* anti-inflammatory assay was tested using 2-[2-(2, 6-dichloroanilino) phenyl] acetic acid (diclofenac), a standard non-steroidal anti-inflammatory drug. The results reveal that there is a gradual increase in percentage inhibition as concentration increases for the crude extracts. The data obtained for extract for both cyclooxygenase and lipoxygenase assays were comparable to the standard drug employed. The half maximal inhibitory concentration also substantiates these results. This is in accordance with the results of Leelaprakash [23]. It can also be authenticated from the results that the ethanol extract can be used as an anti-inflammatory agent as the percentage inhibition was comparable to the standard non-steroidal drug used.

With the aforesaid data, the development of novel, effective and safe anti-inflammatory therapeutics from medicinal plants is achievable. This has been a major research area in the development of non-steroidal anti-inflammatory drugs. Anti-inflammatory agents with selective cyclooxygenase and lipoxygenase inhibition are more appreciated as safe drugs, as they have minimum gastrointestinal side effects. Research reports have proved that naturally occurring coumarins and flavonoids act as dual inhibitors of cyclooxygenase and lipoxygenase activities [24]. Plant derived compounds have to be authenticated and it is also necessary to check its safety, efficacy and stability to be used as crude drugs. Hence, in this scenario, this work can help in the standardization of botanicals in isolating major compounds, especially alkaloids possessing anti-inflammatory activity. Nevertheless, it can also solve the dilemma which needs to be addressed to the consumers for their satisfaction and for the commercialization of the drugs from plant origin. The innocuous nature of plants addresses the safety issue of these botanicals on health grounds.

5. Conclusions

In conclusion the results of the present work may invigorate the process of standardization of botanicals in *Glycosmis pentaphylla*. Also, from the study, as alkaloids are present in copious amount, a need to isolate this metabolite responsible for the anti-inflammatory activity is essential. These findings can act as an aid for those researchers involved in the field of drug discovery. In most of the situations, the compound/s isolated from plants may not serve as drug, but leads to the development of potential medical agents. As new molecules are rapidly identified having significant anti-inflammatory effects, plants prove as an important agent in the mainstream of anti-inflammatory drug discovery marathon.

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