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ASSESSMENT OF ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITIES OF SECONDARY METABOLITES FROM LEAVES AND FRUIT EXTRACT OF *GYMNOSPORIA MONTANA*

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Keywords:

Gymnosporia montana, Secondary metabolite, Antioxidant study, Anti-inflammatory activity

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ABSTRACT: Objectives: *Gymnosporia montana* (family *Celeastraceae*), important shrub which is widely reported in Gujrat, India. In this study, our major objective is to screen secondary metabolites of methanolic leaves and fruit extract and determining its antioxidant and anti-inflammatory potential of this shrub. **Materials and Methods:** Leaves and fruit extract were subjected to methanol followed by phytochemical investigations and estimating the presence of secondary metabolites and also analysing its activity for antioxidant (DPPH and hydrogen peroxide scavenging methods) and anti-inflammatory potentiality were carried out. **Results:** Preliminary studies related to its phytochemical screening of leaves and fruit extract of *Gymnosporia montana* in the form of methanol extract which revealed its existence of flavonoids, alkaloids and terpenoids. In comparison with alkaloid and terpenoid extract, there is significant enhancement in antioxidant activity of flavonoid content in case of methanolic extract in a dose-dependent manner. Thereafter, itsanti-inflammatory activity test was carried out against Typhoid vaccine (25 µg/ml, 10 µl) with these extracts (flavonoids, terpenoids and alkaloids of methanol) using variable concentration and compared with Typhoid vaccine, used as standard. All these secondary metabolites extracts showed significant (especially seen in case of flavonoid from methanolic leaves and fruit extract) decline in antigen specific proliferationas compared to control, but there are no significant activities were observed in terpenoid and alkaloid. **Conclusion:** Overall, this study may indicate that methanol extract having secondary metabolite (flavonoid content) having antioxidant and anti-inflammatory properties.

INTRODUCTION: The usage of these medicinal plant products which is directly correlated with antibiotics and other modern drugs. So, these plant derived products have been effective sources of chemotherapeutic agents without showing any side effect. In literature, several plants were evaluated for various immunobiological activities against various chronic diseases [1, 2].

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Recently, scientists more focused on natural occurring antioxidants (containing free radical scavengers) that are reported in foods and vegetables because of the replacement of synthetic antioxidants having adverse reactions. So, these natural occurring antioxidants is directly associated with several molecules (*i.e.* phenols and flavonoids) that are present in medicinal plant [3, 4].

In contrast, natural antioxidants may be able to protect the human body from free radicals and decline in the progression of various chronic diseases. In addition, India is richly with diverse medicinal plants having anti-inflammatory activities and used as traditional medicine.

The most striking feature about these medicinal plants related to its immunobiological and therapeutic applications [5]. *Gymnosporia Montana* (family, *Celeastraceae*) is widely distributed throughout the world and reported in dry areas of India (Punjab and Gujarat), Afghanistan, Africa and Australia. In this plant, flowers and fruits will appear or grown only when hot and long summer season are required. In literature, several compounds were reported [6, 7] *viz*. tingenone, 3-Oacetyloleanolic acid, hexacosane, hexacosanol, ntriacontanol, betulin, β -amyrone, β-amyrin, δamyrin, β - sitosterol, celacinnine and kaempferol from the leaves of *Gymnosporia montana.* In addition, fruits (2 or 3 valved), globuse capsule (10-20 mm long and 8-9 mm diameter; purplish or black in colour) and its seeds are brownish white with green and fleshy cotyledons [8-10]. In this study, our major objective is to screen its antioxidant and anti-inflammatory activity of leaves and fruit extract from *Gymnosporia montana* with the aim of evaluating its traditional or therapeutic use.

MATERIALS AND METHODS:

Collection and Identification of Plant Material: Leaves and fruit extracts of *Gymnosporia montana* (Latitude: 21º44'36.2" N and Longitude: 72º07'41.4" E) were collected from Victoriya Park, Bhavnagar, Gujarat, India during October 2021. The plant material was authenticated by Dr Naresh Chavda and Mr. Dr SK Mehta. The plant samples were submitted and stored as herbarium in Department of Botany, Maharaja Krishna Kumar Sinhji Bhavnagar University, Bhavnagar (Herbarium No: 01/2022).

Preparation of Extracts: Leaves and fruit extract of the *Gymnosporia montana* were shade-dried for several days and prepared course powder. Take 250 g of the sample (leaves/fruit extract) were successively extracted using solvent system *i.e.* methanol. The reflux method was used separately for extract (after proper drying of each extraction) and finally calculated its yield after solvent removal using rotary evaporator (45°C) and then cool down the extract and stored in a refrigerator (4-5°C) for further investigations.

Phytochemical Analysis and Isolation of Secondary Metabolite: The preliminary phytochemical analysis of methanol extract of *Gymnosporia montana* (leaves and fruit extract) were performed using the standard protocol to identify the presence of alkaloids (using diluted HCl and Wagner's reagent), flavonoids (diluted NaOH) and terpenoids (Chloroform and Sulphuric acid). Based on its existence, we isolate the alkaloid, flavonoid and terpenoid from methanolic (leaves and fruit) extract of *Gymnosporia montana.*

Alkaloid: Take (1 g) of leaves/fruit extract were extracted with ethanol (80%) and then macerated it at room temperature. Therefore, extract of leaves/fruit extract were made alkaline using strong ammonia (pH 9-10). Finally, solution of each extract were further extracted using chloroform and then soak it using distilled water. Therefore, chloroform was evaporated in solution using rotary evaporator and finally getting crude extract *i.e.* alkaloids. For its detection, using silica gel precoated in TLC plate (worked as stationary phase) where solvent system (i.e. toluene: ethyl acetate, 9:1) as mobile phase. Afterwards, developed the plate and then dry at room temperature. Finally, sprayed with Bouchardat's reagent in TLC plate for visualizing the spot [11].

Flavonoid: Dried powdered leaves and fruit extract of *Gymnosporia montana* (2 g) were taken using Soxhlet apparatus having ethanol (70%, v/v) and then allowed to dryness. After drying of leaves and fruit extract in hot air oven (40–45 °C), stored or packed in an air tight box in refrigerator at 5 °C. Finally, residue was marked as hydro-ethanolic extract of *Gymnosporia montana*. Dried leaves and fruit extract of *Gymnosporia montana* (2 g) were extracted successively and finally squashed with distilled water to get respective extracts [12] and then analysed its spot through TLC.

Terpenoid: Dried leaves powder and flower extract (2 g) of *Gymnosporia montana* were extracted using soxhlet apparatus (methanol, 500 ml). Afterwards, dried methanolic extract was dissolved in a minimum amount of methanol and 10g of silica gel was added to it. It was further dried in a desiccator to remove completely the traces of the solvent. The dried material was then packed (wet packing) on top of a short column of silica gel (100g) and then the column was developed with petroleum ether followed by petroleum ether with benzenein the ratio of 3:1.

The fractions are washed with methanol [13]. The TLC studies were executed through silica Gel-G (stationary phase) in the chromatographic plates (15x5 cm, 3 mm thickness) and then used different mobile phases and confirmed its spot.

FTIR Analysis: The FT-IR spectra of active secondary metabolite especially flavonoid from leaves and fruit extract of *Gymnosporia montana* were recorded or evaluated in Perkin Elmer Spectrum Version 10.03.09 using KBr pellet method [14].

Antioxidant Assay: For determining its antioxidant activity, major assays were used and applied in case of leaves and fruit extract (aqueous/methanolic/ethyl acetate).

DPPH (1, 1 – diphenyl - 2 - picryl - hydrazyl) free Radical Scavenging Assay: Antioxidant activity of flavonoid, terpenoid and alkaloid in case of leaves and fruit extract of *Gymnosporia montana* was assessed through DPPH free radical assay [15]. In this assay, reaction mixture consists of alkaloid / flavonoid / terpenoid sample (0.5ml), absolute ethanol (3 ml) and DPPH radical solution (0.3 mL in ethanol, 0.5 mM). In contrast, blank (sample, 0.5 ml and ethanol, 3.3 ml); control (ethanol, 3.5 ml and DPPH, 0.3 ml) samples were used. So, its scavenging activity percentage (AA%) was determined on the basis of this equation-

Scavenging activity = 100 (Absorbance of Sample - Absorbance of blank) / Absorbance of Control

Total Antioxidant Capacity (TAC) Assay: In this assay, we determined its total antioxidant capacity [15] of alkaloid / flavonoid / terpenoid in case of leaves and fruit extract of *Gymnosporia montana*. The method is totally based on its reduction of phosphomolybdic acid [Mo (VI)] to phosphomolybdenum [Mo (V), a blue complex] by the leaves and fruits extract (alkaloid / flavonoid / terpenoid), reference standard (ascorbic acid) and blank (distilled water).

In this assay, solution absorbance of each extract including standard was measured in triplicate, using UV-visible spectrophotometer at 695 nm. Plot the standard curve of the ascorbic acid concentration-absorbance plot (standard curve) and the results were expressed as gAAE/100 g ascorbic acid.

Anti-Inflammatory Activity: In this assay, consent letter was taken from healthy volunteers regarding anticoagulant (EDTA) whole blood samples for analysing its anti-inflammatory activity against specific vaccine antigen. In this assay, human whole blood samples were taken and mixed in similar quantity of sterilized phosphate buffered solution. Centrifuging (3500 rpm; 10 min) these samples and then collect the pellet (containing RBCs).

So, these pelleted cells were taken for estimating its anti-inflammatory activity against Typhoid vaccine (25 µg/ml; 10 µl) using variable concentrations of alkaloid / flavonoid / terpenoid (15.6-500 µg/ml) from leaves and fruit extract of *Gymnosporia montana*. All these samples were maintained at room temperature for about 30 min and then centrifuging at 3000 rpm for 10 min. Finally, haemoglobin content was estimated in pellet containing cells and measured through spectrophotometer at 570 nm [16].

Statistical Analysis: Applied one-way ANOVA test (*P<0.05; **P<0.01 and ***P<0.001)

RESULTS:

TLC Analysis: TLC of all samples were analysed, after spraying with particular reagent, showed spots using UV light at 365 nm. From these studies, it may indicate the presence of different compounds having its Rf values were recorded. In alkaloid, 5 spots were observed and its retention factor (0.24, 0.28, 0.47, 0.76 and 0.85) were calculated. In contrast, extracts in the form of flavonoids were analysed by means of TLC using different mobile phases (n-butanol: acetic acid: water, 2:2:6) with varying concentration. The dark colour bands were observed with its retention value (0.54, 0.69 and 0.84). Similarly, in terpenoid, Different mobile phases were used *i.e.* petroleum ether: ethyl acetate, 4:1 having retention factor i.e. 0.74, 0.79 and 0.82; Benzene: ethyl acetate, 97:5 with retention factor 0.84 and 0.72 for the identification of terpenoids. Finally, blue colour spots were appeared after sprayed with vanillin- phosphoric acid reagent and then heated at 110°C for 5 min.

FTIR Analysis : Analysis of secondary metabolite especially flavonoids from leaves and fruit extract were obtained through FT-IR for analysing the

content on the basis of functional group as shown in **Fig. 1**. These studies may have confirmed its existence of flavonoids from leaves and fruit extract of *Gymnosporia montana.*

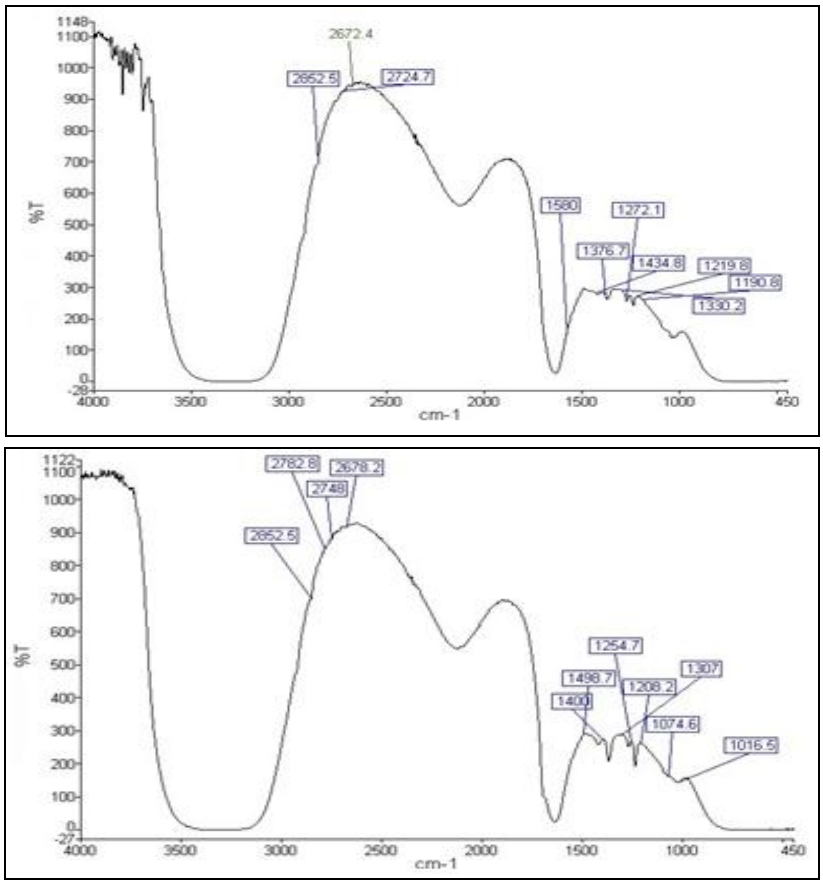

FIG. 1: FTIR ANALYSIS OF METHANOLIC (FLAVONOID CONTENT) FROM LEAVES AND FRUIT EXTRACT. A) Flavonoid leaves extract; B) Flavonoid, fruit extract

Antioxidant Activity: The results showed the effectiveness of flavonoids from leaves and fruit extract of *Gymnosporia montana* as antioxidants as compared to alkaloid and terpenoid extract **Fig. 2**. In this assay, flavonoids exhibited a wide range of radical scavenging activity as compared to alkaloid and terpenoid extract. In this study, *Gymnosporia montana* and ascorbic acid (reference standard) scavenged DPPH at concentrations ranging between 6.25 µg/ml to 800 µg/ml as shown in **Fig. 2**. The DPPH scavenging based studies may have revealed that flavonoids (leaves/fruit extract) of *Gymnosporia montana* may be useful for drug manufacturing pertaining to cure health issues and its problems that may arise from the systemic actions of oxidative agents.

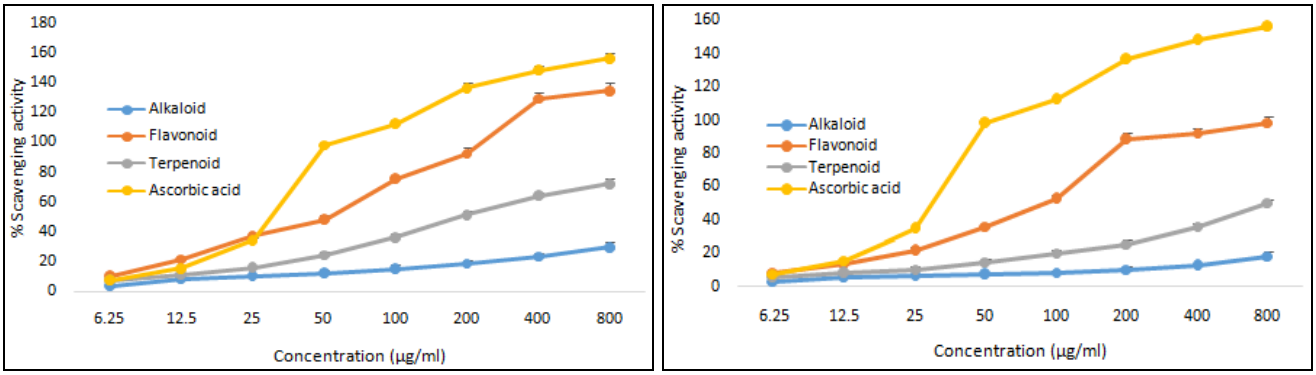

FIG. 2: COMPARATIVE DPPH RADICAL SCAVENGING ACTIVITY OF METHANOL (LEAVES AND FRUIT) EXTRACT AND ASCORBIC ACID. (*P<0.05; **P<0.01 and ***P<0.001

In this assay, flavonoid from leaves and fruit extract of *Gymnosporia montana*was directly proportional to TAC. The results of these studies showed that flavonoid exhibited a drastic enhancement as compared to alkaloid and terpenoid in TAC (examined by the phosphomolybdenum method) expressed as ascorbic acid equivalent per 100 grams (gAAE/100 g; represents plant extract act as ascorbic acid in 100 g of the extract) **Table 1**.

TABLE 1: TOTAL ANTIOXIDANT CAPACITY OF METHANOLIC (LEAVES AND FRUIT) EXTRACT OF *GYMNOSPORIA MONTANA*

S. no.	Samples	TAC (gAAE/100 g)
	Methanolic leaves extract	
1	Alkaloid	14.32 ± 1.04
2	Flavonoid	39.8 ± 1.44**
3	Terpenoid	22.2 ± 1.76
	Methanolic fruit extract	
4	Alkaloid	12.66 ± 0.42
5	Flavonoid	35.24 ± 0.78**
6	Terpenoid	19.64 ± 1.34

Anti-inflammatory Activity: The results of anti-inflammatory based studies of leaves and fruit extract of *Gymnosporia montana* against Typhoid vaccine as shown in **Fig. 3**. The results may indicate its effectiveness of flavonoid from leaves and fruit extract as anti-inflammatory agents against Typhoid vaccine was still higher as compared to the alkaloid and terpenoid.

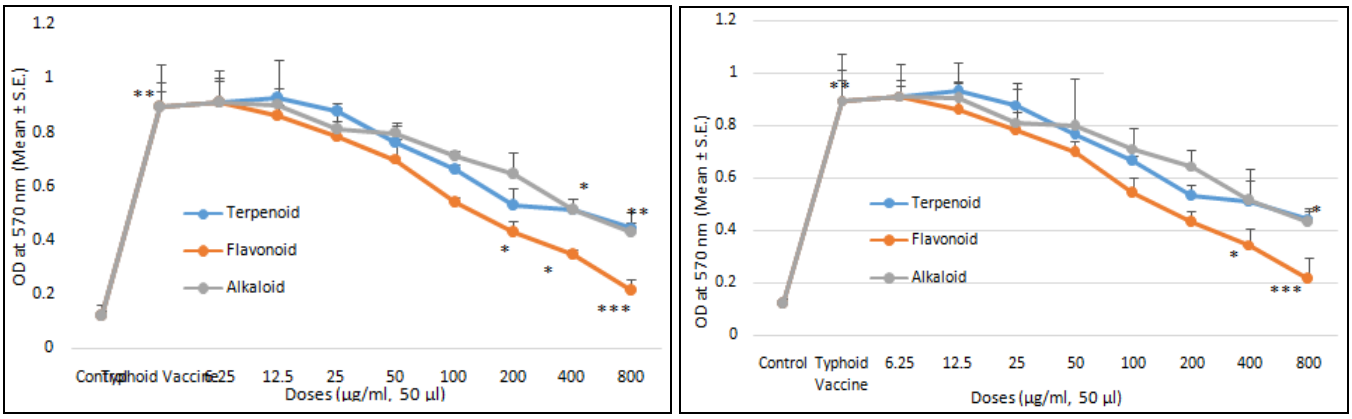

FIG. 3: ANTI-INFLAMMATORY ACTIVITY OF LEAVES AND FRUIT EXTRACT OF *GYMNOSPORIA MONTANA*AGAINST TYPHOID VACCINE. (*P<0.05; **P<0.01 and ***P<0.001.

DISCUSSION: In *Gymnosporia montana*, preliminary investigation of secondary metabolites from leaves and fruit extract revealed the existence of alkaloid, flavonoids and terpenoid. So, these phytochemicals especially secondary metabolites are known to exhibit a wide range of immuno-pharmacological activities *i.e.* antidiabetic, anthelminthic, antimalarial, and many others [8, 9]. In other words, phytochemicals in leaves and fruit extract of *Gymnosporia montana* could be responsible for its traditional therapeutic benefits. In order to achieve this objective, our major objective is to screen the secondary metabolite *(i.e.* flavonoid, terpenoid and alkaloid from leaves and fruit extract) and determined its antioxidant and anti-inflammatory activity. In literature, various techniques were applied for estimating antioxidant activity in plant extracts. The most commonly used methods (i.e. scavenged DPPH radical and phosphomolybdenum method) were applied for measuring its activity. This activity is totally based on DPPH scavenging through the addition of a radical species or antioxidant that decolourizes the DPPH solution. So, degree of colour change is directly proportional to the concentration and potency of the antioxidants [17, 18]. In other words, there is decline in the absorbance of the reaction mixture may indicate significant free radical scavenging activity of the plant extract. In this study, flavonoid content showed significant enhancement in in terms of free radical scavenging property and also represents drastic enhancement in

TAC content as compared to alkaloid and terpenoid. The results of these studies may suggest that *Gymnosporia montana* contain phytochemical constituents especially secondary metabolites in leaves and fruit extract that are capable of donating hydrogen to a free radical to scavenge the potential damage. In addition, another activity related to antioxidants were also evaluated through spectro-photometrically using phosphomolybdenum method. The results also suggest that flavonoids showed highest antioxidant capacity as compared to alkaloids and terpenoids for phosphomolybdate reduction.

In terms of flavonoid content, highest yield was obtained in leaves extract as compared to fruit extract so there was a poor correlation between two of them in *Gymnosporia montana.* In literature, natural occurring flavonoids are reported in plants and showed its positive impact on human health in terms of antibacterial, antiviral, anti-inflammatory, anticancer and anti-allergic activities [19, 20]. So, these flavonoids considered as one of the most effective scavengers of most oxidizing molecules, including singlet oxygen, and various free radicals implicated in several diseases. So when we compare our findings in the literature for other extracts of plant products, our results suggested that flavonoids (category of phenolic compounds) may be the major contributors for the antioxidant activity.

In literature, these medicinal plants containing some vital phytoconstituents that possess various immunopharmacological properties especially anti-inflammatory [21]. Since these medicinal plants are natural, easily available and having no side effects.Another objective of our study was to evaluate the anti-inflammatory activity of secondary metabolite (alkaloid, flavonoid and terpenoid) using typhoid vaccine as antigen. Therefore, flavonoid from leaves and fruit extract of *Gymnosporia montana*with promising inhibitory activity against typhoid vaccine antigen as compared to alkaloid and terpenoid. So, these crude extracts of flavonoids may contain some compounds which is responsible for higher activity as compared to alkaloid and terpenoid. Overall, these results suggest that flavonoid from *Gymnosporia montana* having both antioxidant and anti-inflammatory activities.

CONCLUSION: In this study, we find out its antioxidant and anti-inflammatory activity of flavonoid content is much more as compared to alkaloid and terpenoid. Further studies are still in progress pertaining to compare its antioxidant, antimicrobial and HPTLC analysis of various fractions (n-hexane, ethyl acetate and n-butanol) of methanol extracts obtained by Soxhlet extraction technique from leaves of *Gymnosporia montana.*

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CONFLICTS OF INTEREST: NIL

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