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Evaluation of immunomodulatory activity of hydroalcoholic extracts of *Diospyros melanoxylon* fruits

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Abstract:

Fruits of *Diospyros melanoxylon* (Tembar) were collected from local reasion Mudakhed, Bhokar, Kinwat (Nanded) Golatgaon. Shadedried &Powdered *Diospyros melanoxylon* fruits (100 gm.) were extracted using a Soxhlet extractor sussesively & with hydroalcoholic solvent system in the ratio 1:1, temperature set at 60-70 °C. Hydroalcoholic extract of *Diospyros melanoxylon fruits* preliminary phytochemical analysis was done using standard methods. phytoconstituents were isolated i.e., gallic Acid [3,4,5-trihydroxybenzoic acid] from n- butanol hydroalcoholic extract of fruits of *Diospyros melanoxylon* (NHDM). Phytoconstituent isolation is an important concluding result of the study and cardioprotective activity of the two extracts may be attributed to these phytoconstituents. The hydroalcoholic extract of fruits of *Diospyros melanoxylon* was evaluated for immunomodulatory activity in laboratory animals namely anti-inflammatory activity using carrageenan induced paw edema model, immunomodulatory activity using carbon clarence test, immunomodulatory activity using E. coli induced abdominal sepsis (Determination of Host Resistance). The extract showed potent immunomodulatory activity. The multistep putative action of these plants may be attributed to the prominent phytoconstituents namely i.e., gallic Acid [3,4,5-trihydroxybenzoic acid] from n- butanol hydroalcoholic extract of fruits of *Diospyros melanoxylon* (NHDM).

Keywords: *Diospyros melanoxylon* (Tembar), fruits, Soxhlet extractor, immunomodulatory activity.

Introduction

The term "immunomodulation" refers to the modification of body's immune response which may either decrease (immunosuppression) or increase (immunostimulation) immune responsiveness. An immunomodulator is usually a synthetic or biological agent with an ability to suppress, stimulate or modify any of the mechanisms of the immune system including both humoral or innate immune system [1]

Immunomodulators are biological response modifiers; exert their effects by improving host defense mechanisms. They also enhance the ability of the host to tolerate damage by toxic chemicals that may be used to destroy cancer. Immunomodulatory therapy could provide an alternative to conventional chemotherapy for a variety of diseased conditions, especially when host's defense mechanisms have to be activated under the conditions of impaired immune responsiveness or when a selective immunosuppression has to be induced in a situation, like inflammatory diseases, auto-immune disorders ad organ/bone marrow transplantation [2]

There are various side effects are associated with the use of these drugs i.e. pulmonary toxicity, myelosuppression, alopecia, increased risk of infection, hepatic fibrosis, lymphoma (Epstein–Barr virus associated), nephrotoxicity, neurotoxicity (tremor, headache, motor disturbances and seizures), GI complaints, hypertension, hyperkalemia, hyperglycemia, and diabetes, Renal dysfunction, tremor, hirsutism, hypertension, hyperlipidemia, gum hyperplasia, hyperuricemia, hyper cholesterolemia, nephrotoxicity, hypertension, diabetogenic, elevated LDL cholesterol etc. [3, 4]

Plant extracts used in traditional therapy are being reviewed for their chemo protective and immunomodulatory activities. According to the World Health Organization (WHO), about three-quarters of the world population relies upon traditional remedies (mainly herbs) for the health care of its people. (REF) A number of Indian medicinal plants and various Rasayana have been claimed to possess immunomodulatory activity [5]

Diospyros melanoxylon is one of such medicinal plants which has been documented to possess anti-ulcer, antihyperglycemic, anticancer and analgesic activities and has been traditionally claimed to possess immunomodulatory activity but has not been yet documented scientifically. In light of this, the present investigation was focused on the evaluation of immunomodulatory activity of hydroalcoholic extracts of *Diospyros melanoxylon* fruits.

Material and Methods:

Plant material:

Fruits of *Diospyros melanoxylon* (Tembar) were collected from Mudakhed, Bhokar, Kinwat (Nanded) Golatgaon (Aurangabad) also procured from a local vendor and were authenticated by Dr. Marathe V. R. Asst. Prof. Department of Botany, Science College Nanded affiliated with SRTMU.

Animals:

Swiss albino mice (20-40gm) and Wistar albino rats (180-220 gm) of either sex was procured from Local vendor and were maintained at 25 ± 2° C and relative humidity of 45 to 55% and under standard environmental conditions (12 h light: 12 h dark cycle). The animals had free access to food and water throughout study. Institutional Animal Ethical Committee approved the protocol. All the experiments were carried out between 9:00- 16:00 hour.

Extraction of selected plant material:

Powdered *Diospyros melanoxylon* fruits (100 gm.) were extracted successively using a Soxhlet extractor with hydroalcoholic solvent system is used in the ratio 1:1 and the temperature was set at 60-70 ºC. Extract was filtered, concentrated and after complete solvent evaporation, each of these solvent extracts was weighed and preserved at 5°C in an airtight bottle until further use. Hydroalcoholic extract of *Diospyros melanoxylon fruits* was prepared and labeled as HEDM.

Preliminary phytochemical analysis of extract:

The hydroalcoholic extract of *Diospyros melanoxylon fruits* was subjected to preliminary phytochemical analysis for the presence of prominent chemical constituents using standard methods [6, 7]

Acute oral toxicity study of extract:

Acute oral toxicity study of hydroalcoholic extract of *Diospyros melanoxylon* fruits (HEDM) was carried out as per OECD guideline 425 [8, 9]

Evaluation of anti-inflammatory activity of hydroalcoholic extracts of fruits of *Diospyros melanoxylon* fruits (HEDM) using carrageenan induced paw edema model:

30 Wistar rats of either sex weighing between 200-250g were used. The over-night fasted animals were divided into six groups (n=6) as follows:

Group-1: Animals of this group received distilled water (1 ml/kg, p.o.)

Group-2: Animals of this group received HEDM (100 mg/kg, p.o.)

Group-3: Animals of this group received HEDM (200 mg/kg, p.o.)

Group-4: Animals of this group received HEDM (400 mg/kg, p.o.)

Group-5: Animals of this group received indomethacin (400 mg/kg, p.o.)

These rats were subjected to respective treatments as per the groups for the period of 14 days. On the 14th day, 1 hour after the dosing, all rats were injected with 0.1 ml of 1% carrageenan into sub plantar region of hind paw and paw volume (mean displacement Volume) was measured at 0th 3rd, 6th and 24th hour using digital plethysmometer.

Evaluation of immunomodulatory activity of hydroalcoholic extracts of fruits of *Diospyros melanoxylon* fruits (HEDM) using carbon clearance test

24 Wistar rats of either sex weighing between 200-250g were used. The over-night fasted animals were divided into six groups (n=6) as follows:

30 Wistar rats of either sex weighing between 200-250g were used. The over-night fasted animals were divided into six groups (n=6) as follows:

Group-1: Animals of this group received distilled water (1 ml/kg, p.o.)

Group-2: Animals of this group received HEDM (100 mg/kg, p.o.)

Group-3: Animals of this group received HEDM (200 mg/kg, p.o.)

Group-4: Animals of this group received HEDM (400 mg/kg, p.o.)

Phagocytic activity of reticuloendothelial systems (RES) was assayed by carbon clearance test [9,10]

Phagocytic index was calculated as a rate of carbon elimination of reticuloendothelial systems by carbon clearance test. The rats were subjected to respective treatments as per the groups for the period of 14 days. On the 14th day, 1 hour after the dosing, carbon ink suspension was injected via the tail vein to each rat 48 hours after the five-days treatment. Blood samples (25 µl) were then withdrawn from the retro-orbital plexus under mild ether anesthesia at 0 and 15 minutes after injection of colloidal carbon ink and lysed in 0.1% sodium carbonate solution (03 ml). The optical density was measured spectrophotometrically at 660 nm. The phagocytic index was calculated using the
following formula;
K = log OD 1 – log OD 2 / t 2 – t 1
Where, OD1 and OD2 are the optical densities at time t 1 and t 2.

Evaluation of immunomodulatory activity of hydroalcoholic extract of fruits of *Diospyros melanoxylon* fruits (HEDM) using *E. coli* induced abdominal sepsis (Determination of Host Resistance)

40 rats divided into four groups each consisting of 08 rats (here n=6 was taken considering mortality).
Group-1: Animals of this group received distilled water (1 ml/kg, p.o.)
Group-2: Animals of this group received HEDM (100 mg/kg, p.o.)
Group-3: Animals of this group received HEDM (200 mg/kg, p.o.)
Group-4: Animals of this group received HEDM (400 mg/kg, p.o.)
Group-5: Animals of this group received Imipenam 20mg/kg
Rats of different groups were subjected to respective treatments as mentioned in following table for the period of 14 days.
On 14th day, 1 hour after dosing all rats were injected with 1.0 mg of *E. coli* serotype 0111:B4 LPS in a volume of 500µl of sterile solution and were observed for mortality for next 72 hrs with the interval of every 24 hours.
After completion of 72hrs, 10ml of sterile saline was injected into the abdominal cavity. 30 minutes later, a fifty micro liters of peritoneal lavage fluid from each survived rat were collected, placed on ice and serially diluted with sterile saline. Twelve micro liters of each dilution were spread on sterile nutrient agar plates and incubated overnight at 37°C after which the number of colonies was counted and expressed as CFU/ml of E coli.[10]

Statistical Analysis:

Data obtained was subjected to statistical analysis of suitable parametric or non-parametric test using Graph pad Instat software, USA.

Phytochemical qualitative analysis of extracts

The hydroalcoholic extract of *Diospyros melanoxylon* fruits was subjected to phytochemical qualitative analysis for presence of various phytoconstituents of extracts.

High Performance Thin Layer Chromatography (HPTLC) studies of hydroalcoholic extract of *Diospyros melanoxylon* fruits

Instrumentation: HPTLC system of CAMAG, Muttenz, Switzerland, Anchrom Enterprises (I) Pvt. Ltd, Mumbai, consisting of sample applicator (Linomat 5), Twin trough chamber with

lid {10×10 cm, CAMAG, Muttenz, Switzerland), UV cabinet (Aetron, Mumbai) with dual wavelength (254/366 nm) was used for study.

Chromatographic Conditions: The sample (Hydroalcoholic Extract) was applied in the form of band of width 6 mm with a 100 µL sample syringe (Hamilton, Bonaduz, Switzerland) on precoated silica gel aluminium plate 60 F254 (5 ×10) with 250 µm thickness (E. MERCK, Darmstadt, Germany) using a CAMAG Linomat 5 sample applicator (Switzerland). The plate was prewashed with methanol and activated at 110 0C for 5 minutes, prior to chromatography. The optimized chamber saturation time for mobile phase was kept 15 min. The length of chromatogram run was 8 cm. HPTLC plate was dried in a current of air with the help of a hair dryer. The slit dimensions of 5× 0.45 mm and scanning speed of 20 mm/sec were employed in analysis.

Mobile phase: The composition of mobile phase was Chloroform: Methanol (8:2)

Calculation of Rf Values: Plate was observed in the daylight, under UV light (254 and 366 nm). After each observation the central points of spots appeared on chromatogram were marked with needle. Retention factor (Rf) was calculated by following formula[7, 11]

Rf = A/B

A = distance between point of application and central point of spot of material being examined.

B = distance between the point of application and the mobile phase front.

RESULTS

Preliminary phytochemical analysis of extract:

The hydroalcoholic extract of fruits of *Diospyros melanoxylon* (HEDM) showed the presence of saponins, tannins, terpenoids, flavonoids, alkaloids.

Acute oral toxicity study of extract:

The hydroalcoholic extract of *Diospyros melanoxylon* fruits (HEDM) did not show any signs of morbidity nor mortality and any type of adverse effects found up to the dose of 2000 mg/kg p.o.

Evaluation of anti-inflammatory activity of hydroalcoholic extracts of fruits of *Diospyros melanoxylon* fruits (HEDM) using carrageenan induced paw edema model:

Table 1. Evaluation of anti-inflammatory activity of hydroalcoholic extracts of fruits of *Diospyros melanoxylon* fruits (HEDM) using carrageenan induced paw edema model:

Sr. No.	Treatment	0 min	3 hrs	6 hrs	24 hrs
1	Normal control	1.57±0.034	1.85±0.025	2.47±0.020	2.64±0.020
2	HEDM (100 mg/kg)	1.53±0.025	1.79±0.018	2.37±0.014*	2.54±0.017*
3	HEDM (200 mg/kg)	1.54±0.027	1.77±0.016*	1.99±0.031**	2.55±0.022*
4	HEDM (400 mg/kg)	1.54±0.027	1.66±0.012**	1.94±0.038**	2.13±0.021**

5	Indomethacin (400 mg/kg)	1.55±0.022	1.60±0.022**	1.74±0.029**	1.62±0.024**

Values are mean ± S.E.M., n=6; Statistical analysis by one-way ANOVA followed by Dunnett's test using Graph pad Instat software; $p<0.05$*, $p<0.01$**and $p<0.001$*** compared to control group.

At zero-minute no significance difference observed in paw edema of all treated group as compared to control. At 03 hours the higher two doses of extract showed dose dependent inhibition in the paw edema as exhibited through the volume of displacement. The highest dose is equipotent to the reference standard. At 06 hours all doses of extract showed significant reduction however upper two doses were equipotent and more significant than lowest dose. At 24 hours the lowest dose and highest dose of the extract has shown same activity as that of 6th hour but middle dose has shown reduction in its action. This is to be related to potency and duration of action. Overall, as far as both onset potency and duration of action is considered, then highest dose of the extract was found to be most effective.

Evaluation of immunomodulatory activity of hydroalcoholic extracts of fruits of *Diospyros melanoxylon* fruits (HEDM) using carbon clarence test

Evaluation of immunomodulatory activity of hydroalcoholic extracts of fruits of *Diospyros melanoxylon (HEDM)* using carbon clarence test

Table 2. Evaluation of immunomodulatory activity of hydroalcoholic extracts of fruits of *Diospyros melanoxylon (HEDM)* using carbon clarence test

Sr. No.	Treatment	Phagocytic index
1	Normal control (Isotonic NaCl solution, 10mg/kg)	1.168±0.017
2	HEDM (100 mg/kg)	1.158±0.012
3	HEDM (200 mg/kg)	1.156±0.012
4	HEDM (400 mg/kg)	1.277±0.021**

Values are mean ± S.E.M., n=6; Statistical analysis by one-way ANOVA followed by Dunnett's test using Graph pad Instat software; $p<0.05$*, $p<0.01$**and $p<0.001$*** compared to control group.

In this study only 400 mg/kg dose of the extract showed significant ($P<0.01$) increase phagocytic index as compared to normal control

Evaluation of immunomodulatory activity of hydroalcoholic extract of fruits of *Diospyros melanoxylon* fruits (HEDM) using E. coli induced abdominal sepsis (Determination of Host Resistance)

Table 3. Evaluation of immunomodulatory activity of hydroalcoholic extracts of fruits of *Diospyros melanoxylon (HEDM)* using *E. coli* induced abdominal sepsis (Determination of Host Resistance)

Sr. No.	Treatment	Phagocytic index
1	Control (isotonic NaCl solution, 10mg/kg)	121.66±3.83
2	HEDM 100mg/kg	119.33±1.89
3	HEDM 200mg/kg	115.83±1.37
4	HEDM 400mg/kg	108.83±1.99**
5.	Imipenam 20mg/kg	105.16±2.35**

Table 4. Survival of rats after E coli challenge

Groups (n=8)	Survival after 24 hrs	Survival after 48 hrs	survival after 72 hrs
Control (isotonic NaCl solution, 10mg/kg)	6/8	4/8	4/8
HEDM 100mg/kg	6/8	5/8	4/8
HEDM 200mg/kg	6/8	5/8	5/8
HEDM 400mg/kg	7/8	6/8	6/8
Imipenam 20mg/kg	8/8	7/8	7/8

Wherever due to mortality, the number of animals have reduced from 6 (n=6) then average reading (if it's less by 1) and repetition of highest and lowest reading(if it's less by 2) was done to get statistical comparison.

In this study, only 400 mg/kg dose of the extract showed significant ($P<0.01$) and equipotent activity as that of standard drug imipenem as compared to normal control.

Statistical Analysis:

Data obtained was subjected to statistical analysis of suitable parametric or non-parametric test using Graph pad Instat software, USA.

Phytochemical qualitative analysis of extracts

High Performance Thin Layer Chromatography (HPTLC) studies of hydroalcoholic extract of *Diospyros melanoxylon* fruits

A

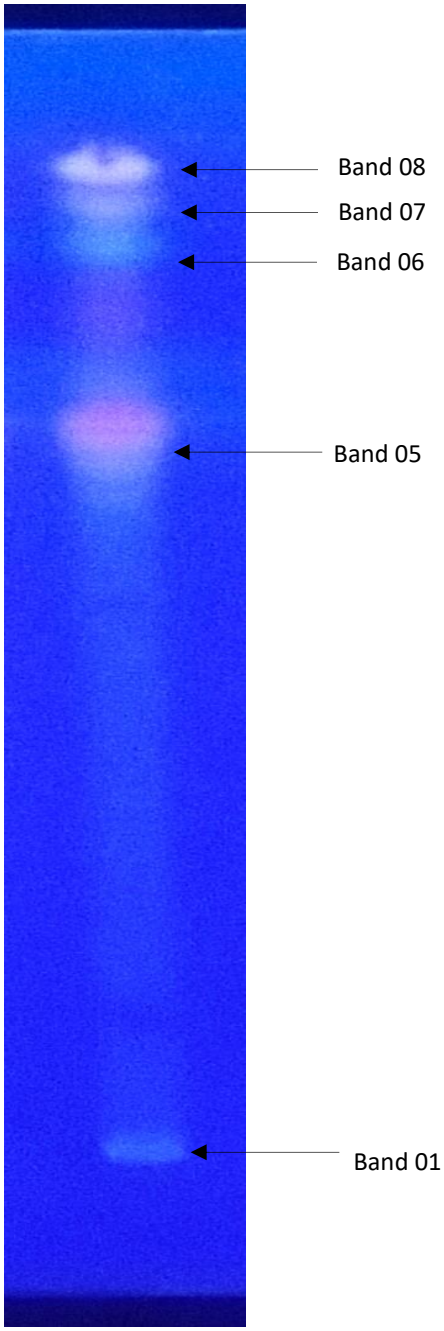

Figure 1. TLC plate n Butanol fraction of Hydroalchoholic extract of *Diospyros melanoxylon* fruits at 366 nm, Volume applied 10 µl.

The Band 5 at Rf Value 0.77 was scratched and subjected to structure elucidation. B B

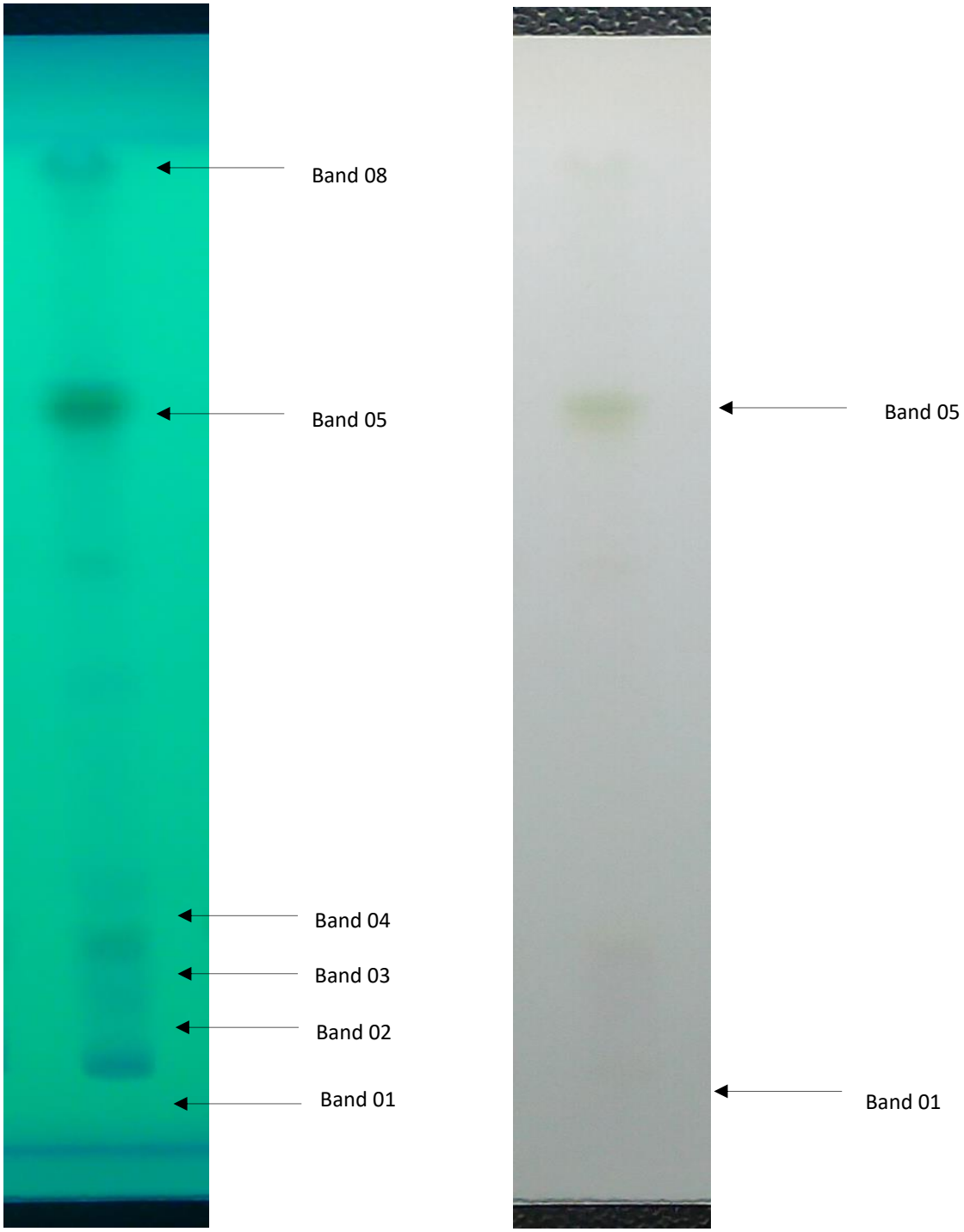

Figure 2. TLC plate of n Butanol fraction of Hydroalchoholic extract of *Diospyros melanoxylon* fruits at 254 nm, Volume applied 10 µl.

The Band 5 at Rf Value 0.77 was scratched and subjected to structure elucidation.

The Band 5 at Rf Value 0.77 was scratched, extracted with methanol and evaporated to dryness for further analysis by IR, NMR and Mass Spectrometry.

Spot at Rf Value – 0.77

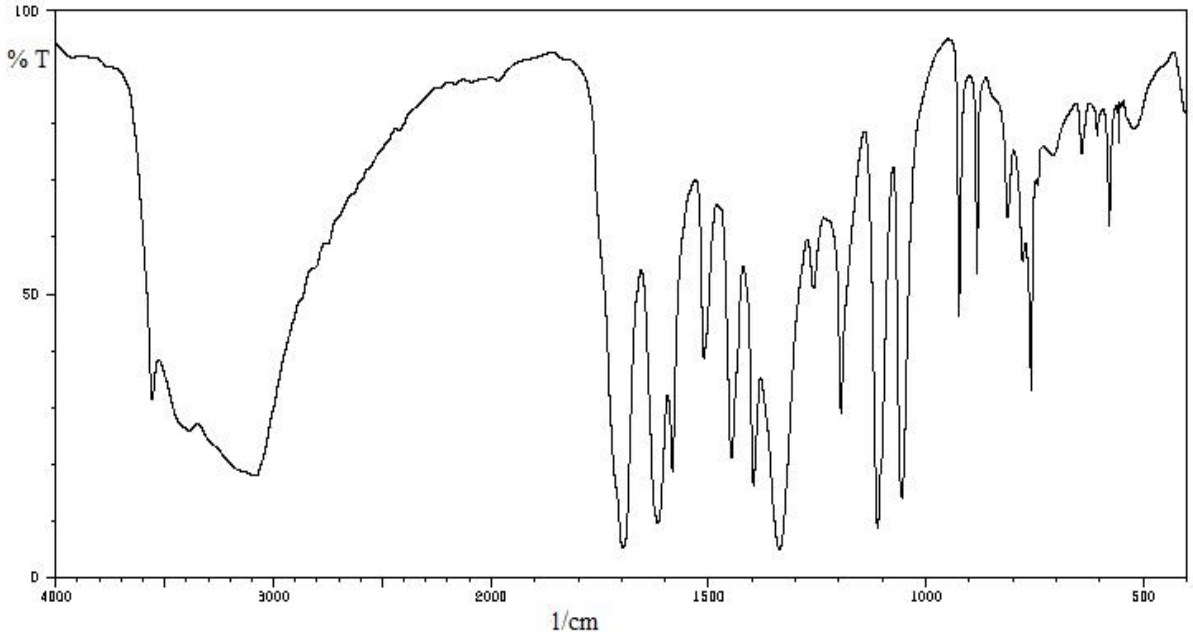

Figure 3. FT-IR Spectrum of compound at Rf – 0.77

Table 5. Interpretation of IR Spectrum

Sr. No.	Part of molecule	Vibration	General Range (Cm^{-1})	PI 36
1	Ar Rings	a) C=C stretch b) C-H stretch c) C-H bend d) Overtone	1500-1650 3000-3100 740-762 1700-2000	1644 3053 757 1700-2000
2	-O-H	O-H stretch	3200-3600	3344
3	C=O	C=O stretch	1650-1750	1712

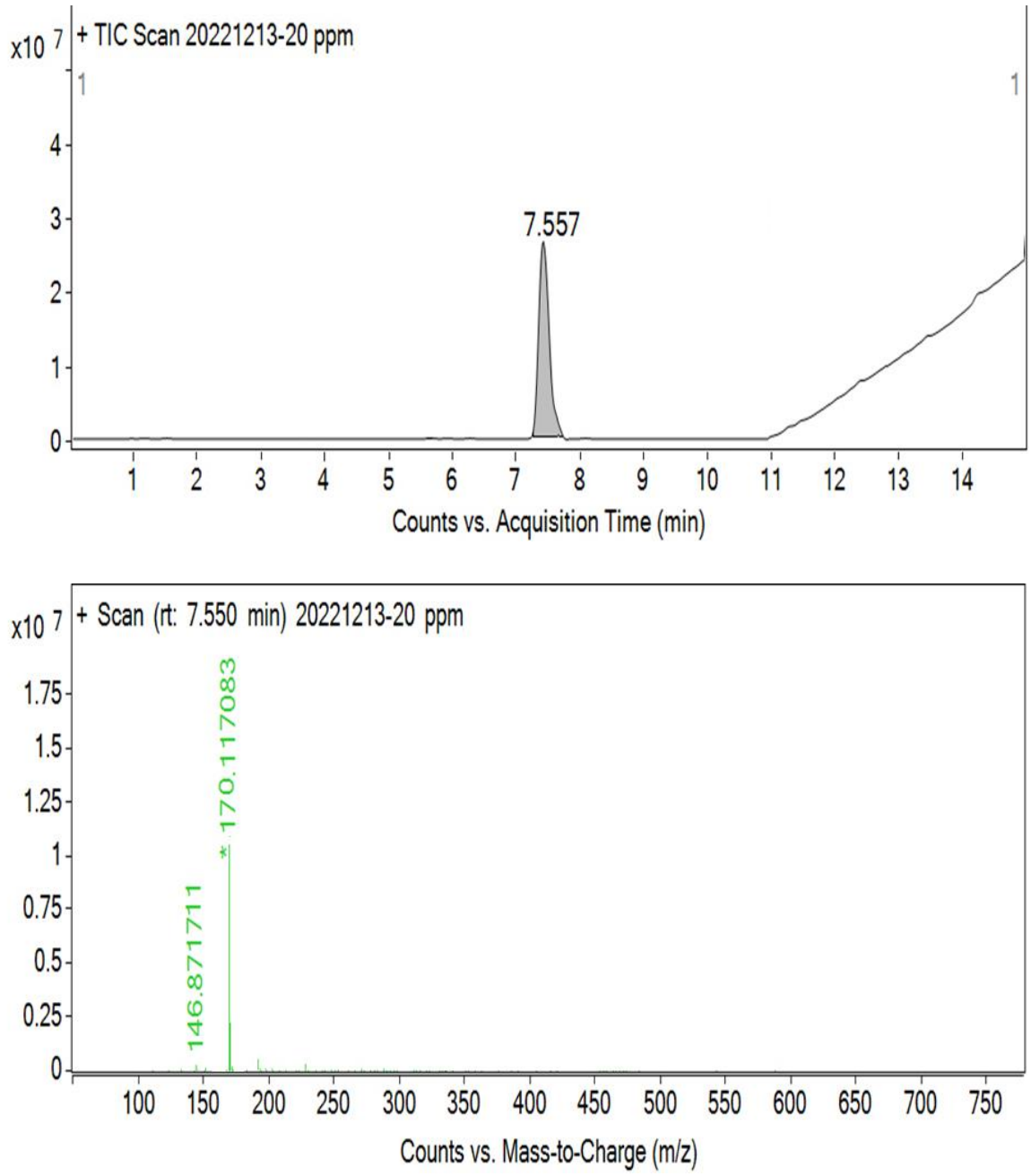

Figure 4 LC-MS A) chromatograph and B) Spectrum of compound at Rf – 0.7

Table 6. Interpretation of NMR spectrum

Sr No.	δ	No of Protons (Multiplicity)	Type
1	2.62	1 H (s)	OH Proton (p-position)
2	3.14	2 H (s)	02 OH Protons (m-position)
3	6.87	2 H (s)	02 Aromatic protons
4	11.82	1 H (s)	COOH Proton

Probable structure of the isolated compound is as below

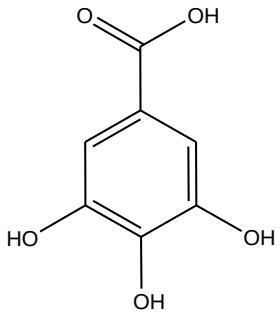

Figure 5. Structure of gallic Acid [3,4,5-trihydroxybenzoic acid]

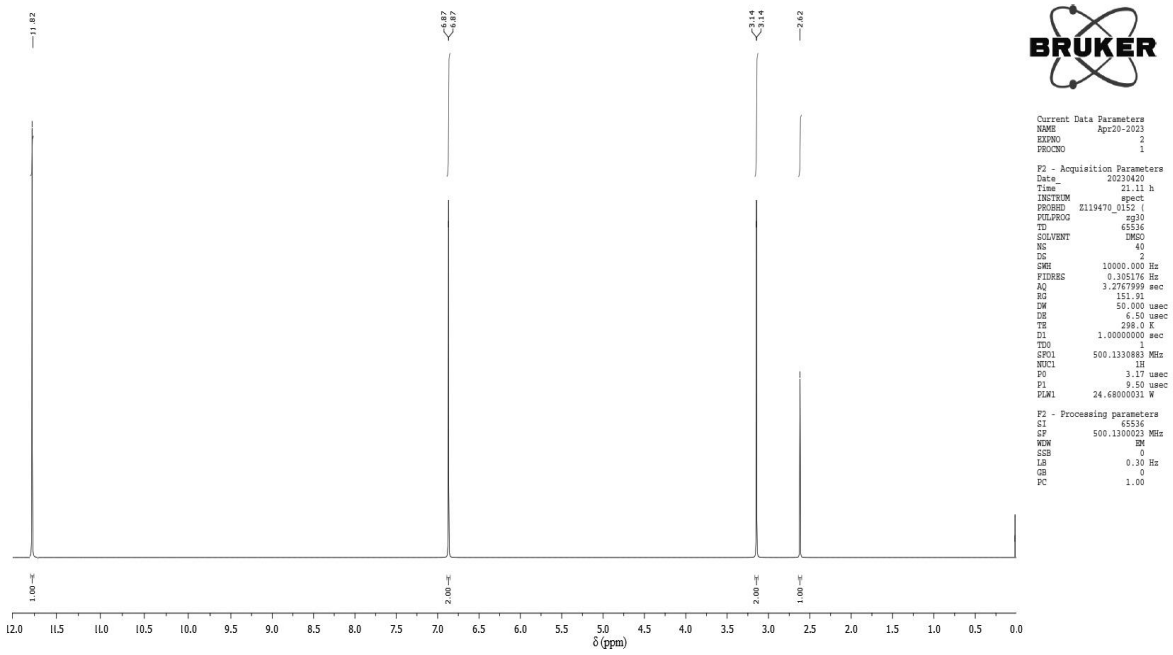

Figure 6. NMR Spectrum of compound at Rf – 0.77

DISCUSSION:

Immunomodulators are agents that aid in immune system regulation. The concept of immunomodulation relates to nonspecific activation of the function and efficiency of macrophages, granulocytes, complement, natural killer cells and lymphocytes and also to the production of various effector molecules generated by activated cells. It is expected that theses nonspecific effects give protection against different pathogens including bacteria, viruses, fungi etc., and constitute an alternative to conventional chemotherapy [12]

In recent years, there has been a surge of interest in herbal medicine research and the quest for intriguing prospective chemicals for studying immunomodulatory substances derived from natural sources. The immune system is intended to defend the host against invading microorganisms and to eradicate illness [13] . Plants play an important role in Complementary and Alternative Medicine (CAM) because of their ability to produce secondary metabolites such as proteins, flavonoids, alkaloids, steroids, and phenolic substances, which are then used to restore health and heal many diseases[14] . Herbal medications are thought to boost the body's natural resistance against infection, and immunomodulatory properties have been discovered in a variety of plants. The primary techniques by which herbs fight the body against illness may be divided into two categories: killing pathogens and increasing bodily immunity [15]

Diospyros melanoxylon is one of such such herbal medicines with wide range of pharmacological actions. It has been traditionally claimed to possess beneficial effects on the immune system but has not been yet scientifically documented for its immunomodulatory effects [16,17,18,19]

Hence the present investigation “Preclinical evaluation of the medicinal plant *Diospyros melanoxylon* for its immunomodulatory activity in laboratory animals” was carried out.

The immunomodulatory activity of herbal medicine and the mechanisms therein depends on the phytoconstituents present in them. The actual therapeutic results are closely related to the conventional wisdom, scientific findings, and phytochemical presence. The most trustworthy way to determine whether phytoconstituents are present is by a preliminary phytochemical examination, followed by a comparison of the results with existing literature [20] . So as to assess the phytochemical profile, the hydroalcoholic extract of fruits of *Diospyros melanoxylon* (HEDM) was subjected to preliminary phytochemical analysis. Results of the preliminary phytochemical analysis revealed that hydroalcoholic extract of fruits of *Diospyros melanoxylon* (HEDM) showed the presence of saponins, tannins, terpenoids, flavonoids, alkaloids. These phytoconstituents have been reported to possess immunomodulatory effects [21,22,23] . Natural products may be a source in the quest for immunomodulatory medicines due to the evaluation and confirmation of these activities of several phytochemical ingredients in preclinical models [21] . The plants quality, particularly in relation to the phytoconstituents responsible for its toxicity profile and immunomodulatory effect, has not yet been properly proven. Toxicology profile testing becomes extremely important to ensure its safety before moving forward with the actual exploration of its pharmacological activity after the phytochemical profile and its pharmacological importance have been confirmed. To confirm the extent of a therapeutic drug's therapeutic use, toxicity profile testing is crucial for all therapeutic drugs [24] . Moreover, in the present investigation, the results of preliminary phytochemical analysis suggested further pharmacological exploration of extract, hence it was essential to assess the extract for its toxicity profile and thereby confirmation of its safety [8] . Hence the acute toxicity study of the

hydroalcoholic extract of fruits of *Diospyros melanoxylon* (HEDM) was carried out as per OECD guideline 425. The results of acute oral toxicity studies revealed that the extract was safe with no signs of morbidity nor mortality and any type of adverse effects up to 2000 mg/kg which is highest prescribed limit as per this test. Thus, the extract fulfilled the safety criteria before the assessment of preclinical activity (OECD guidelines 425). Based upon these findings and available literature, the three different doses i.e. 100, 200 and 400 mg/kg of the extract were selected for the further preclinical investigations.

In the present study, the immunomodulatory effects of the extract was evaluated using three widely used preclinical screening models namely anti-inflammatory activity using carrageenan induced paw edema model, immunomodulatory activity using carbon clarence test, immunomodulatory activity using *E. coli* induced abdominal sepsis (Determination of Host Resistance). These models are known to be of highly predictive relevance with respect to the clinical spectrum of activity [25, 26, 27].

Carrageenan-induced paw edoema is a well-defined model of acute inflammation that includes a range of inflammatory mediators in its development and has been widely utilised to assess the anti-edematous activity of natural products. In nature, the development of carrageenan-induced paw edoema is a biphasic process. Histamine, serotonin, and bradykinins are released from mast cells into the surrounding injured tissues during the initial phase (1-2 hours after carrageenan injection). The production of arachidonate metabolites such as prostaglandins, leukotrienes, and different cytokines such as IL-1, IL-6, IL-10, and TNF- is related with the second phase of the inflammatory reaction (3-6 h after carrageenan injection) [25, 28, 29].

In anti-inflammatory activity using carrageenan induced paw edema model, the extract showed significant and dose dependent reduction in the paw edema at 3, 6 and 24 hours indicating potent anti-inflammatory activity in second phase of inflammation indicating inhibitory effect against several inflammatory mediators. The results were found to be comparable to that of the standard drug indomethacin.

The carbon clearance test is usually done to evaluate the effect of drugs on the reticulo-endothelial system. The reticuloendothelial system (RES) is a diffuse system consisting of phagocytic cells. Cells of the RES play a vital role in the clearance of particles from the bloodstream. When colloidal carbon particles in the form of ink are injected directly into the systemic circulation, the rate of clearance of carbon from the blood by macrophage is governed by an exponential equation [26]. In the carbon clearance test, the extract showed marked increase in the phagocytic index as compared to the normal control at the higher dose (400 mg/kg) indicating immunostimulant activity of the extract. These effects were found to be equipotent to the standard drug imipenem.

In the face of bacterial infection, the innate immune response is a crucial line of defence. In the late stages of sepsis, however, increasing immunosuppression impairs innate immune activity [29]. While the first immune response is critical for successful pathogen clearance, an excessively enthusiastic host response to infection can result in septic shock, tissue damage, and death. Sepsis is typically accompanied by widespread immunosuppression, which increases susceptibility to viral and bacterial infections [29, 30]. LPS is a major sepsis-inducing component of Gram-negative bacteria, such as *Escherichia coli*.

In the *E. coli* induced abdominal sepsis test, the extract showed marked decrease in the colony forming units (CFU/ml) of *E. coli* and increased survival of rats after *E coli* challenge as

compared to the normal control at the higher dose (400 mg/kg) indicating immunostimulant activity of the extract. These effects were found to be equipotent to the standard drug imipenem. Many of the pharmaceuticals that are currently on the market have either been directly or indirectly produced from plants, which have traditionally been exceptional sources of drugs. These phytopharmaceuticals have offered many advantages over the current drugs from modern medicine however availability of drug and variation regarding the yield as well as quality with respect to cultivation from time to time, season, region etc. cannot be ignored [31]. The requirement of large amount of crude drug to obtain the desired quantity of extract is another major drawback of herbal medicine. Utilizing medicinal biodiversity to its fullest extent in order to search for phytochemicals with immunomodulatory properties is a difficult task. Additionally, administration of phytoconstituents without desired pharmacological activity led to precipitation of adverse effects or reduced therapeutic efficacy [11] .

This problem can be overcome by the isolation of most active phytoconstituent responsible for the desired pharmacological activity from the extract. This not only identifies and isolates the active phytoconstituent but also provides an important input to the synthetic chemistry regarding the structure of patient friendly bioactive constituent with required pharmacological action[32] . HPTLC technique is most simple and fastest separation technique available today which gives better precision and accuracy with extreme flexibility for various steps [11] . In light of this, n- butanol fraction of hydroalcoholic extract of fruits of *Diospyros melanoxylon* (NHDM) were subjected to the High-Performance Thin Layer Chromatography (HPTLC) studies.

As a result of these studies phytoconstituents was isolated i.e., gallic Acid [3,4,5-trihydroxybenzoic acid] from n- butanol hydroalcoholic extract of fruits of *Diospyros melanoxylon* (NHDM).

The isolation of the phytoconstituent is an important concluding result of the study and cardioprotective activity of the two extracts may be attributed to these phytoconstituents.

Conclusion

The present study documented the immunomodulatory activity of the medicinal plants *Diospyros melanoxylon*. The hydroalcoholic extract of fruits of *Diospyros melanoxylon* and was evaluated for immunomodulatory activity in laboratory animals namely anti-inflammatory activity using carrageenan induced paw edema model, immunomodulatory activity using carbon clarence test, immunomodulatory activity using E. coli induced abdominal sepsis (Determination of Host Resistance).

The extract showed potent immunomodulatory activity. The multistep putative action of these plants may be attributed to the prominent phytoconstituents namely i.e., gallic Acid [3,4,5-trihydroxybenzoic acid] from n- butanol hydroalcoholic extract of fruits of *Diospyros melanoxylon* (NHDM).

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