

IN VITRO ANTIOXIDANT AND ANTIBACTERIAL ACTIVITY OF ESSENTIAL OIL FROM THE SEEDS OF NEOLITSEA PALLENS (D. DON) MOM & HARA

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ABSTRACT

The essential oil from the seeds of *Neolitsea pallens* (D. Don) Moms & Hara growing in the Himalayan region is evaluated for its antioxidant activity using test for phenolic, flavanoid content and ferric reducing/antioxidant power assay (FRAP) and antibacterial activity by well diffusion method. The oil has shown 356.1 ± 0.003 mg/ml gallic acid equivalent per 100mg of the oil as total phenolic content and 355 ± 0.014 mg/ml quercetin equivalent per 100 mg of the oil as total flavonoid content. The oil exhibited marked antioxidant activity. The VEAC values were found to be 0.454g/L and 0.538g/L for the essential oil and ascorbic acid respectively. The ZOI for the test bacterial strains at a concentration of 500 μ g/ml were Gram negative bacteria

*Pseudomonas aeruginosa*424 (7mm), *Escherichia coli*443 (4mm), *Aeromonas hydrophila*646 (4mm) and Gram positive bacteria *Bacillus subtilis*441 (4mm) and *Staphylococcus aureus*737 (6mm).

KEYWORDS: *Neolitsea pallens*, essential oil, antioxidant, antibacterial activity, infections.

1. INTRODUCTION

Medicinal plants found wide use as alternative therapeutic tools for the control, treatment and prevention of several diseases.^[1] The medicinal and aromatic plants have been traditionally used as medicines and preservatives for food stuff as they inhibit the growth of bacteria, fungi and yeasts.^[2] *Neolitsea* belongs to family Lauraceae. Some of the plants from the genus *Neolitsea* have been used as folk lore medicines for a long time in Subtropical regions of

Asia, there are more than 2500 species belonging to the Lauraceae family all over the world, distributed within the subtropics and tropics of eastern Asia and South and North America.^[3] Plants having significant amount of phenolic compounds have attributed various activity, the most relevant of which is antioxidant activity, which is important in countering oxidative stress which arises mainly as a consequence of the over production of free radicals due to imbalance in production of antioxidants by the cells.^[4] Several studies revealed that phenols, mainly the type of flavonoids, from some medicinal plants are safe and bioactive and have antioxidant properties and exert anticarcinogenic, antimutagenic, antifumoral, antibacterial, antiviral and anti-inflammatory effects.^[5] Some of the sesquiterpenes from the plants of the genus *Neolitsea* showed significant effects on platelet aggregation induced by arachidonic acid, collagen and platelet activating factor (PAF) in vitro.^[6,7] Phytochemical studies on the plants of *Neolitsea* led to the Identification and Isolation of 183 compounds which have been found to be rich in alkaloids, sesquiterpenoids and triterpenoids with bioactivity.^[8] In human Body, several reactive oxygen species (ROS) such as hydrogen peroxide, superoxide anion radical and hydroxyl radical are produced through oxygen consumption by several enzymatic systems.^[9] The protection against the damage caused by uncontrolled production of reactive oxygen is provided by antioxidants.^[10] The leaf extract of *N. Parvigemma* showed significant antioxidant activity ($IC_{50} = 5.73 \mu\text{g/ml}$) in the DPPH assay.^[11] It is possible to minimize the risk of chronic diseases and prevent disease progression by supplementing with proven dietary antioxidants.^[12] Bacteria are responsible for several infections and ailments in all living organisms including human beings. *Aeromonas hydrophila* reports from Australia have suggested that there may be connection between cases of *Aeromonas* associated diarrhea and the members of *Aeromonas* in the drinking water.^[13] *Bacillus subtilis* are spore producing bacteria which are found in the gastrointestinal tract of Human beings and other animal, responsible for producing several infections and ulcers of GIT.^[14] The present study reveals antioxidant and antibacterial activity of natural products (seed oil) from *N. pallens*.

2. MATERIALS AND METHODS

2.1. Plant material

The Plant material (seeds of *Neolitsea pallens*) were collected from Munsaiyari area of Uttarakhand (2400m-2600m) during month of October-November when the fruits were in their ripening stage. The Plant specimen was primarily identified by Prof. P. C. Pandey, Botany Department, Kumaun University, Nainital. The botanical identity was further confirmed by Botanical Survey of India, Dehradun, Voucher specimen [*Neolitsea pallens* (D.

Don) Moms & Hara, Acc. No. 115215] have been deposited in the Herbarium of Botanical Survey of India, Northern Regional Centre, Dehradun (BSD).

2.2. Extraction of the Essential oil

The essential oil was obtained by hydro-distillation of fresh seeds (200gm) by Clevenger. The oil was extracted with Hexane and Ether. The solvent extract was dried using anhydrous sodium sulphate (Na_2SO_4) and the solvent was removed with Rotovap at aspirators pressure and at 36°C to yield 4.1gm of oil (2.05% by weight). Residual oil was stored at 4°C till further analysis. All chemicals and reagents used were of analytical grade. Hexane, ether, anhydrous sodium sulphate (Na_2SO_4), and Dimethyl Sulphoxide(DMSO) were obtained from Merk, Mumbai, India were as potato dextrose agar (PDA), Potato dextrose broth (PDB) were obtained from Himedia, India.

2.3. Determination of total Phenolic content (TPC)

The total phenolic content in the essential oil was determined by Folin-Ciocalteu's method.^[15] with certain modifications. 1ml of the test sample was mixed with 1ml Folin-Ciocalteu's reagent and 0.8ml Na_2CO_3 (7.5%). The Mixture was kept at room temperature for 30 min and then absorbance was measured at 765 nm. Quantification of total phenolic content was based on standard curve of Gallic acid prepared in the two solvents respectively. The results were expressed in mg Gallic acid equivalent (GAE) per gram dry weight of the material.

2.4. Determination of Total Flavonoid Content

The total flavonoid contents were determined spectrophotometrically.^[16] In brief, 1ml of the sample was mixed with 75 μl , 5% of NaNO_2 solution. The mixture was allowed to stand at room temperature for 5 minutes. Then 75 μL of $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ (10%) solution was added to it and allowed to stand for 5 minutes. Then 0.5 ml of 1M NaOH solution was added to the above solution and shaken vigorously and allowed to stand for 15 minutes at room temperature. After 15 minutes the absorbance was obtained in spectrophotometer at a Wavelength of 510nm. Quantification of total flavonoid content was done on the basis of standard curve of quercetin prepared in the two solvents and the results were expressed in mg quercetin equivalent (QE) per gram dry weight of the material.

2.5. Determination of antioxidant activity

2.5.1. Determination of Ferric reducing antioxidant power (FRAP) Assay

It is one of the simple, direct tests for measuring of antioxidant capacity. It provides a measure of the reducing ability of the essential oil. The total antioxidant and potential of the oil was determined by using ferric reducing ability (FRAP) assay^[17], as measure of antioxidant power. The FRAP reagent was freshly prepared by mixing 10mM 2,4,6-tripyridyltriazine (TPTZ) and 20mM ferric chloride in 0.25M acetate buffer, pH 3.6. 100µl of the test sample was added to 300µl of distilled water followed by 3ml of FRAP reagent. The absorbance was measured at 750nm after 5 min incubation at room temperature against a blank. The standard curve of tocopherol was constructed. Antioxidant power could be determined from the standard curve as Vitamin E Equivalent antioxidant capacity. Antioxidant activity of the sample was compared with standard ascorbic acid. This assay measures the change in absorbance at 750nm owing to the formation of a blue colored Fe^{2+} -tripyridyltriazine compound from colorless oxidized Fe^{3+} form by the action of electron donating antioxidants. The present data are average of three replications.

2.6. Determination of Antibacterial activity

2.6.1. Source of tested organisms

Pure cultures of all microorganisms were obtained from IMTECH (Institute of Microbial Technology), Chandigarh, India. Microorganisms (bacteria) selected for study were.

Gram negative

Pseudomonas aeruginosa (M.T.C.C. No.424), *Escherichia Coli*(M.T.C.C. No. 443), *Aeromonas hydrophila*(M.T.C.C. No. 646).

Gram Positive

Bacillus subtilis (M.T.C.C. No.441) and *Staphylococcus aureus* (M.T.C.C. No.737).

2.6.2. Preparation of Inoculums

Nutrient agar (NA) and Nutrient Broth (NB) were used for culturing the bacteria. Inoculums were prepared by picking respective bacterial colony with the help of loops and poured into NB then incubated at 28°C for 48 hours for bacteria growth in shaking condition.

2.6.3. Antibacterial activity

The antibacterial activity was determined by using Agar well diffusion method.^[18] It was expressed as the mean of ZOI diameters (mm) produced by the essential oil. For screening plates were prepared by using nutrient agar. The inoculums (90µl) of different bacterial strains were spread evenly on respective plates with sterile spreader and a borer (8mm diameter) was used to cut well. 20µl of different concentrations 5mg/ml, 10mg/ml, 15mg/ml, 20mg/ml (100µg/well, 200µg/well, 300µg/well, 400µg/well) and 25mg/ml (500µg/well) respectively, of the oil were poured in each well and incubated at 28°C for 48hours. Control (Negative control) well contained 20µl of 5% DMSO in place of essential oil solution. The diameter of ZOI was measured and the mean was recorded. Experiment was performed in triplicate.

2.6.4. Statistical Analysis

For all tests, the mean values and standard deviations were calculated. The data were analyzed using SPSS 16.0 statistical software. The one-way analysis of variance (ANOVA) was applied for calculating results. The means were compared by Duncan tests at a level of significance of $P < 0.05$.

3. RESULTS

3.1. Total phenolic contents

Phenolic compounds may directly contribute to the antioxidant activity of the essential oil. The total phenolic content was 356.1 ± 0.003 mg/ml gallic acid equivalent per 100mg of the oil.

3.2. Total flavonoid content

Flavonoids present in the essential oil of the *N. pallens* may also contribute towards antioxidant activity. The total flavonoid content of the oil was 355 ± 0.004 mg/ml quercetin equivalent per 100 mg of the oil.

3.3. Ferric reducing antioxidant power (FRAP) assay

The reducing ability of the essential oil was evaluated on the basis of VEAC (Vitamin E Equivalent Antioxidant Capacity) where the standard curve of the FRAP assay of tocopherol (Vitamin E) was plotted as absorbance (Table 1) versus different concentrations after 5 minutes (Fig.1). The antioxidant potential of the essential oil was assessed in the same way (Fig.2) and compared with that of ascorbic acid (Fig.3). VEAC values were calculated using

appropriate equations and were found to be $0.454 \pm 0.003\text{g/l}$ and $0.538 \pm 0.001\text{g/l}$ for the essential oil and ascorbic acid respectively. The FRAP values for the essential oil are quite compare able to that of the ascorbic acid. The antioxidant power shows good linear relation in both standard ($R^2 = 0.948$) as well as sample oil ($R^2 = 0.926$) (Fig. 3, 2).

Table 1: absorbance shown by the sample and control (Ascorbic acid) at different concentrations.

| Concentration | absorbance | | |
|----------------------|-------------------|-------------------------|------------------|
| | Sample | control (ascorbic acid) | Vitamin E |
| 100 $\mu\text{l/ml}$ | 0.985 ± 0.021 | 0.350 ± 0.001 | 0.875 ± 0.00 |
| 200 $\mu\text{l/ml}$ | 1.785 ± 0.003 | 1.830 ± 0.012 | 2.135 ± 0.00 |
| 300 $\mu\text{l/ml}$ | 1.985 ± 0.01 | 3.960 ± 0.006 | 4.013 ± 0.00 |
| 400 $\mu\text{l/ml}$ | 2.250 ± 0.002 | 4.330 ± 0.014 | 5.673 ± 0.00 |
| 500 $\mu\text{l/ml}$ | 2.574 ± 0.003 | 5.354 ± 0.002 | 7.986 ± 0.00 |

Values in the table are mean of the three results \pm SD.

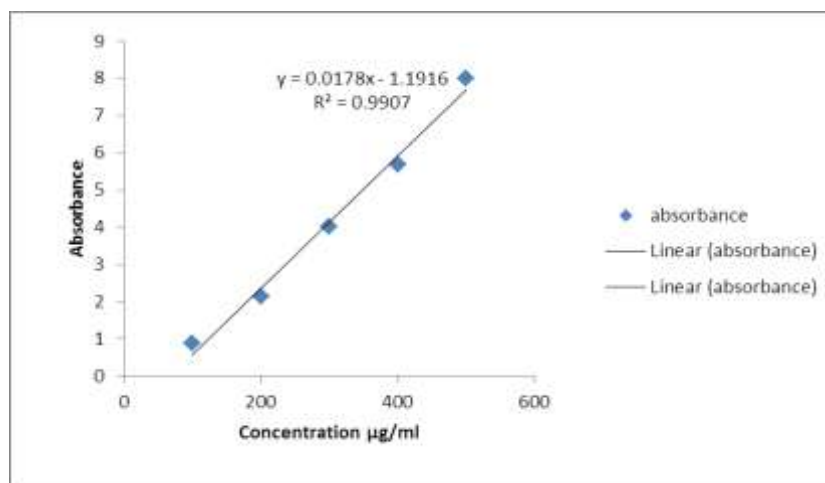


Fig.1: FRAP assay of Vitamin E.

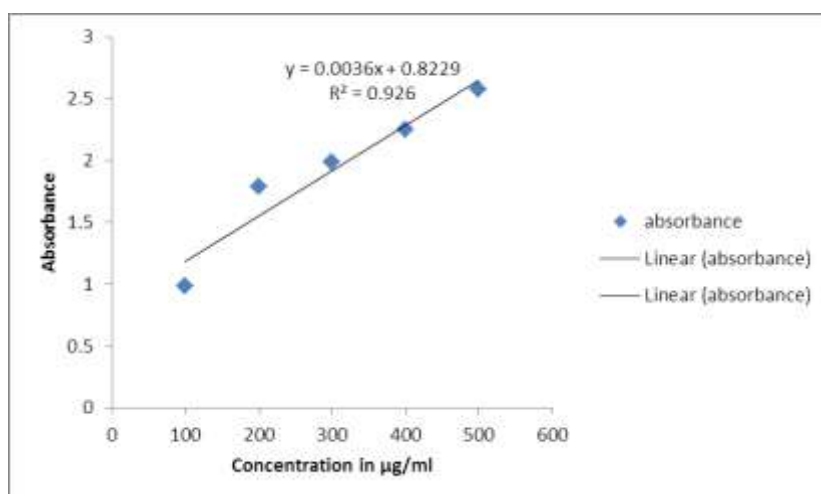


Fig.2: FRAP assay of essential oil from the seeds of *N. pallens*.

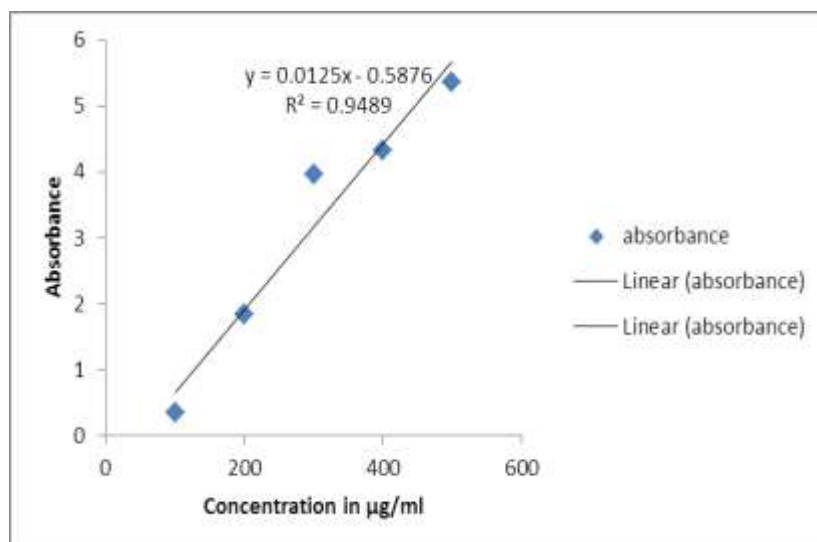


Fig.3: FRAP assay of ascorbic acid.

3.4. Antibacterial activity

Antimicrobials are the agents which kill or inhibit the growth of microorganisms. The antibacterial effects of the natural products from medicinal plants are sufficient in a way to cater the healing effect. The results of the antibacterial efficacy of the oil from the seeds of *N. pallens* as zone of inhibition are presented in table 2. The potency of the oil was tested for both Gram negative and Gram positive bacteria. The maximum ZOI was shown against *P. aeruginosa* (7mm) and *S. aureus* (6mm) at a concentration of 500µg/ml. The oil has shown significant effect against both these strains and lowest effect against *E. coli*. The oil has shown its effect in a dose dependant manner while the control which was 20µl of 10% DMSO only, didn't have good antibacterial activity on these tested bacterial strains.

4. DISCUSSIONS

FRAP assay measures the reducing potential of an antioxidant reacting with a ferric tripyridyltriazine complex and producing a colored ferrous tripyridyltriazine. Generally, the reducing properties are associated with the presence of phenolics, flavonoids and other compounds which exert their action by breaking the free radical chain by donating hydrogen atom.^[19] The relationship between phytochemicals and antioxidants have been assessing for predicting antioxidant property of the plants, the presence of abundant total phenolics in several medicinal plants exhibited high FRAP values.^[20] Consequently, the seed oil from *N. Pallens* results shows exemplary reducing power. Previous reports suggests that, the leaf.

Table 2: ZOI shown by essential oil from the seeds of *N. pallens* against different bacterial strains.

| Conc. → Bacterial strain ↓ | 100µL/mL | 200µL/mL | 300µL/mL | 400µL/mL | 500µL/mL |
|-------------------------------|----------|----------|----------|----------|----------|
| <i>P. aeruginosa</i> | 00mm | 2mm | 2mm | 4mm | 7mm |
| <i>E. coli</i> | 00mm | 1mm | 2mm | 2mm | 4mm |
| <i>A. hydrophila</i> | 00mm | 2mm | 3mm | 3mm | 4mm |
| <i>B. subtilis</i> | 00mm | 1mm | 3mm | 3mm | 4mm |
| <i>S. aureus</i> | 1mm | 3mm | 4mm | 5mm | 6mm |

essential oils from seven Himalayan Lauracere species including *N. Pallens* showed potent antioxidant and antibacterial activities^[21] were as methanolic extracts show strong antioxidant activity.^[22] Subsequent reports also suggests that *Neolitsea* species are found rich in flavonoids which exhibit antioxidant activity^[23] and the essential oil from leaves of *N. Pallens* exhibited potent antioxidant activity, their potent free radical scavenging activity was evidenced by low IC₅₀ values for DPPH radicals (0.032mg/ml and 0.087 mg/ml resp.) And inhibition of lipid per oxidation (IC₅₀ was between 0.44mg/ml and 0.74 mg/ml).^[21] In the present study, the trends for ferric ion reducing activities of seed oil from *N. pallens*, clearly increased, due to the formation of the Fe-TPTZ complex with increasing concentration in a dose dependant manner as standard antioxidant ascorbic acid. Another report suggests that the seed oil of *N. Pallens* was tested against three Gram -ve (*Escherichia coli*, *Salmonella enteric* and *Pasteurella multocida*) and one Gram +ve (*staphylococcus aureus*) bacteria at different concentrations using disc diffusion and tube-dilution methods, the inhibition zone (IZ) and MIC values for bacterial strains were in the range of 8.7mm – 22.0mm and 3.90µl/ml-31.25 µl/ml respectively.^[24] The essential oil (seed oil) from *N. pallens* showed significant effect against all tested strains of bacteria. This property of the oil can contribute towards a substitute for chemical drugs with lesser side effects in comparison to these synthetic medicines and drugs.

5. CONCLUSION

In this present study, promising antioxidant and antibacterial activity has been shown by the essential oil from the seeds of *N. pallens*. These activities are due to presence of total phenolic, total flavonoid compounds present in the crude oil. The results of this study suggest that the essential oil of *N. pallens* can be used as easily accessible source of natural antioxidants, as preservative, as a possible food supplement or in pharmaceutical and

substantial source of multipurpose bioactive principles which act as natural antibiotic against various bacterial strains. However, further molecular investigations in order to isolate the antioxidant and bioactive structures actually responsible for this activity need to be carried out.

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