Evaluation of Immounomodulatory activity of medicinal plant (*Excoecaria agallocha*) by using cyclophosphamide induced neutropenia

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Abstract:

Because the immune system is important in defending humans against alien substances (microorganisms), it must be protected. To function efficiently, immune system cells significantly rely on cell-cell communication, notably through membrane-bound receptors. Since cell membranes contain polyunsaturated fatty acids, peroxidation by reactive oxygen species (ROS) damages the membrane, resulting in a loss of integrity, altered fluidity, and changes to intracellular signalling and cell function. As a result, immune cells that are not appropriately shielded by antioxidants risk causing damage to their own cells. This demonstrates the need for antioxidant molecules. Additionally, numerous biological processes, like ant mutagenicity, ant carcinogenicity, ant ageing qualities, etc., work more effectively when anti antioxidant property is present.

Antioxidants can thus be thought of as being fundamentally essential for life in order to eliminate many pathological conditions and for wellbeing. With their established antioxidant properties, extract from *Excoecaria agallocha* could play a significant role in this aspect. The present work is an effort to reveal the dwindling body of ethnomedical knowledge about greens, with its bioactivity determination and active principle from Excoecaria agallocha, potent antioxidant and immunoprotective agents.

Keywords: *Excoecaria agallocha,* Immunoprotective, Antioxidant.

1.Introduction

Plants have been utilised as medicine since the dawn of time to cure a variety of illnesses. Under the influence of modern medicine, plants have continued to make a significant contribution to the management of health care in recent decades. Medicinal plants are those that are frequently employed in the treatment and prevention of particular afflictions and diseases. They are typically thought to be significant contributors to the field of medicine. The study of medicinal plants has drawn more attention in recent years as new medications are being developed. About 25% of all prescription medications in modern medicine are thought to be derived either directly or indirectly from medicinal plants [1].

According to the World Health Organization (WHO), 65–80% of the world's population lives in developing nations and uses plants as their major source of healthcare because people frequently view them as safer and more affordable than traditional drugs [2]. Plants have a significant part in health management in the well-established traditional medical systems of Traditional Chinese Medicine, Japanese K. ampo, and Indian Ayurveda. As a result, plants are key bioprospecting tools for the identification of new medications [3].

This research work has been performed to evaluate the Immounomodulatory activity of selected medicinal plant by using Cyclophosphamide induced neutropenia.

Material and methods:

1.1 Pharmacognostical Analysis

Identification and collection of the raw material

The fresh leaves of the chosen medicinal plants were collected from Akshat Nursey in Bhopal (M.P.) between March and June 2025. The medicinal plants were chosen with the assistance of local herbal Dealers.

1.2 Extraction [4]

Excoecaria agallocha were two of the plants whose leaves were separated from the fresh and dried on sheets of filter paper in the shade at room temperature until the filter papers' colours changed. The 300g of coarsely powdered, shade-dried materials were defatted using petroleum ether (430C). To obtain hydroxyalcoholic extract, the defatted marc was then treated to a Soxhlet extraction using 70% methanol and water. Different extracts were produced by drying the hydoalcoholic extracts at low temperatures (300C) under reduced pressure, and they were then kept in a refrigerator for additional experimental research.

1.3 Phyto-chemical Screening [5,6,7]

1.3.1 Qualitative test analysis [8]

The tests were performed on the hydoalcoholic extracts in order to identify the various compounds that were present . Alkaloids, glycosides, carbohydrates, tannins, resins, flavanoids, steroids, proteins, and amino acids were all detected using phytochemical screening.

1.3.2 Determination of Total Phenolic Content (TPC) [9]

Using the Folin-Ciocalteu technique, the total phenolic content of all the extracts was calculated. By creating dilutions of (0.8, 1.6, 3.12, 6.23, 12.3, and 23 g/ml) in methanol from a standard gallic acid solution, a standard gallic acid curve was created. Following the addition of 100 l of Folin-Ciocalteu reagent, 100 l of each of these dilutions were combined with 300 l of water and left to stand for 6 minutes.

The reaction mixture was then given an additional 300 l of distilled water and 1ml of 7% sodium carbonate. After 90 minutes, the absorbance was measured spectrophotometrically at 760 nm UV/visible wavelength. With extracts, the same process was repeated. The amount of Gallic acid equivalents (mgGAE/g) used to measure the overall phenolic content of the extracts. Each experiment was carried out twice, and the total phenolic content of the extracts was determined using a regression equation. Every experiment was run twice, and the results are presented as the average of two analyses. Using Microsoft Excel, the degree of correlation between variables was calculated.

1.3.4 Determination of Total Flavonoid Content (TFC)[10]

The flavonoid concentration was determined to be the quercetin equivalent using quercetin as the benchmark. For this, a calibration curve for quercetin was created. The dilutions of (10, 20, 30, 40, and 50 g/ml) concentrations of quercetin were made in methanol from the standard quercetin solution. Following the addition of 100 l of 3% sodium nitrate, 300 l of distilled water, and 100 l of each quercetin dilution, the mixture was left to stand for six minutes. Then, 200 l of a 1M sodium hydroxide solution were successively added after 130 l of a 10% aluminium chloride solution had been introduced and had been left to stand for 3 minutes. On a UV/visible spectrophotometer, the absorbance of this reaction mixture was measured at 310 nm.

The same process was repeated with the extracts, and the amount of total flavonoid content (mgQE/g) was determined. Every procedure was carried out twice. Every experiment was run twice, and the results are presented as the average of two analyses. Using Microsoft Excel, the degree of correlation between variables was calculated.

1.3.5 Antioxidant Activity[11,12]

1.3.6 DPPH Radical Scavenging Ability

DPPH radical scavenging abilities of test plant extracts was carried out according to section.

1.3.7 Superoxide radical scavenging ability

The procedure outlined by Liu et al. (1997)[13] was used to evaluate the test plant extracts' capacity to scavenge superoxide radicals. The production of superoxide radicals by the PMS-NADH system was monitored by reducing NBT. In a 100 mM, 3 ml tris-HCl buffer with a pH of 7.4, 300 mM NBT solution, 936 mM NADH solution, and various quantities of test plant extracts (20, 40, 60, 80, and 100 g/ml), superoxide anion was produced. The reaction was started by adding 750 l of PMS at 120 M concentration. Using a spectrophotometer, the mixture was incubated at room temperature for 5 minutes to measure the absorbance at 560 nm. The positive control was BHT (20, 40, 60, 80, and 100 g/ml), and the negative control was 1% DMSO.

1.3.8 Human erythrocyte haemolysis inhibition ability[14]

For evaluating the test plant extracts' capacity to suppress erythrocyte hemolysis, Gebrelibanos et al(2012) .'s methodology was used. One millilitre of various concentrations of the test plant extracts—20, 40, 60, 80, and 100 g/ml in PBS—was added to two millilitres of a 10% (v/v) solution of erythrocytes in PBS. 2 ml of 100 M H_2O_2 was then added, and the mixture was then incubated at 37 °C for 180 minutes. The reaction mixture

was centrifuged after being diluted with PBS following incubation. The sample's level of hemolysis inhibition was assessed by measuring the reaction mixture's supernatant at 540 nm. Erythrocyte hemolysis brought on by H_2O_2 in the absence of any inhibitors was regarded as 100% hemolysis, and the impact of test plant extracts was calculated using the formula below. The positive control was BHT (20, 40, 60, 80, and 100 g/ml in PBS), and the negative control was 1% DMSO.

1.3.9 Determination of Erythrocytes haemolysis inhibition ability

$$\text{Erythrocytes haemolysis inhibition}\% = \frac{\textit{Absorbance of control} - \textit{absorbance of sample}}{\textit{Absorbance of control}} \text{x100}$$

Where, absorbance of control is the absorbance of suspension of erythrocytes in PBS + $H_2O_2$and absorbance of samples is the absorbance of suspension of erythrocytes in PBS + H_2O_2+ test plant extracts/standard (positive control).

1.3.10 Cyclophosphamide induced neutropenia [15].

Swiss albino mice received the drug or vehicle orally for 10 days. On 10th day, a neutropenic dose of cyclophosphamide (200 mg/kg, sc) was administered and this day was labeled as day zero. Blood samples were collected through retro-orbital vein. The total leucocyte count (TLC) and DLC were performed prior to and on day 3 after injection of cyclophosphamide. The TLC and DLC in treated groups were compared with the values of the control group.

The World Health Organization has developed policies, directives, and standards for botanical medicines in recognition of the value of conventional medicine. Plant-based materials are vulnerable to contamination, degradation, and compositional changes. As a result, procedures for the quick, precise, and accurate identification and estimate of active elements must be developed in order to ensure the consistency of key constituents in formulations (Thakkar et al,.2008).

Results

2.1 Physicochemical properties

Table No. 1: Solvent Extractive Values of Crude Drugs

S. No.	Name of the drug	Water soluble extractive value (% W/W)		Alcohol soluble extractive value (% W/W)	
		Theoretical	Obtained	Theoretical	Obtained
1.	*Excoecaria agallocha*	>10	18.19	>02	11.62

The amount of extractable plant matter can be impacted by temperature and solvent types. The amount of extractive matter produced under a specific condition increases the extractive capability (measured as extractive value). Black pepper's water soluble extractive values must not be less than 3% and 2%, respectively, for water and alcohol soluble extractive values, according to the herbal monograph. The standard specifications were not met by any specimens. According to this study, water as a solvent at higher temperatures had a greater capacity for extraction than alcohol-based solvent at room temperature.

Table No. 2: Physical characteristics of Excoecaria agallocha Extract

S. No.	Name of the Drug	Values	Foreign organic matter	Total Ash value	Acid insoluble ash value	water soluble ash value
1.	*Excoecaria agallocha*	Theoretical	<2%	<5%	<1%	-
		Observed	0.46%	2.31%	0.82%	3.24%

A crude drug's evaluation verifies its identity and establishes its quality and purity. The primary factors necessitating the study of crude drugs include biochemical variations in the substance, therapeutic effects, drug storage issues, adulteration, and replacements (Jarald 2007 and Kadam et al 2012).

2.3 Phytochemical studies

2.3.1 Qualitative phytochemical analysis

Excoecaria agallocha leaf hydoalcoholic extract demonstrated the presence of alkaloids, steroids, terpenoids, saponins, flavonoids, phenols, and glycosides in the qualitative phytochemical study. The specifics of the outcomes are outlined in Table.

Table No. 3: Phytochemical evaluation of *Excoecaria agallocha* Leaves.

S.No.	Phytoconstituents	*Excoecaria agallocha*
		Hydoalcoholic Extract
1	Alkaloids	+ve
2	Steroids/ Terpenoids	+ve
3	Tannins	-ve
4	Saponins	+ve
5	Flavonoids	+ve
6	Phenols	+ve
7	Glycosides	+ve

2.4 Quantitative Analysis

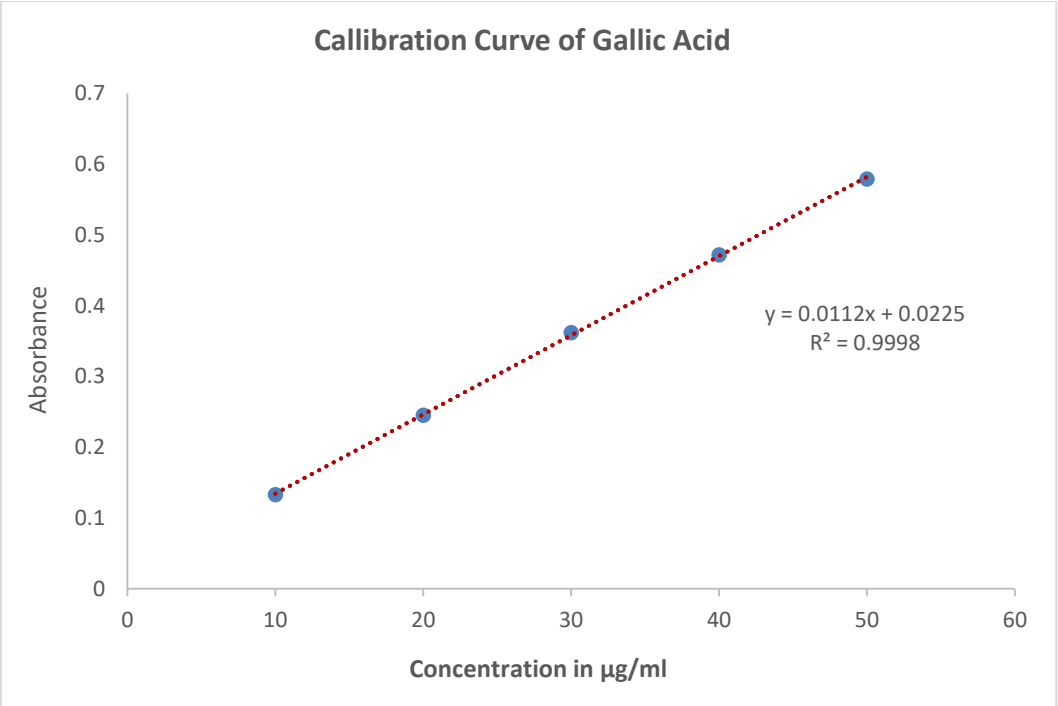
2.4.1 Total Phenolic content estimation (TPC) of *Excoecaria agallocha*

Using the calibration curve's equation: Y = 0.0112X+0.0221, R2= 0.9998, where X is the Gallic acid equivalent (GAE) and Y is the absorbance, total phenolic compounds (TPC) were expressed as mg/100mg of Gallic acid equivalent of dry extract sample.

Calibration Curve of Gallic acid

Table No. 4: Preparation of Calibration curve of Gallic acid

S. No.	Concentration (µg/ml)	Absorbance
1	10	0.133
2	20	0.245
3	30	0.362
4	40	0.472
5	50	0.579
6	Hydoalcoholic extract of *Excoecaria agallocha*(10 µg/ml)	0.412



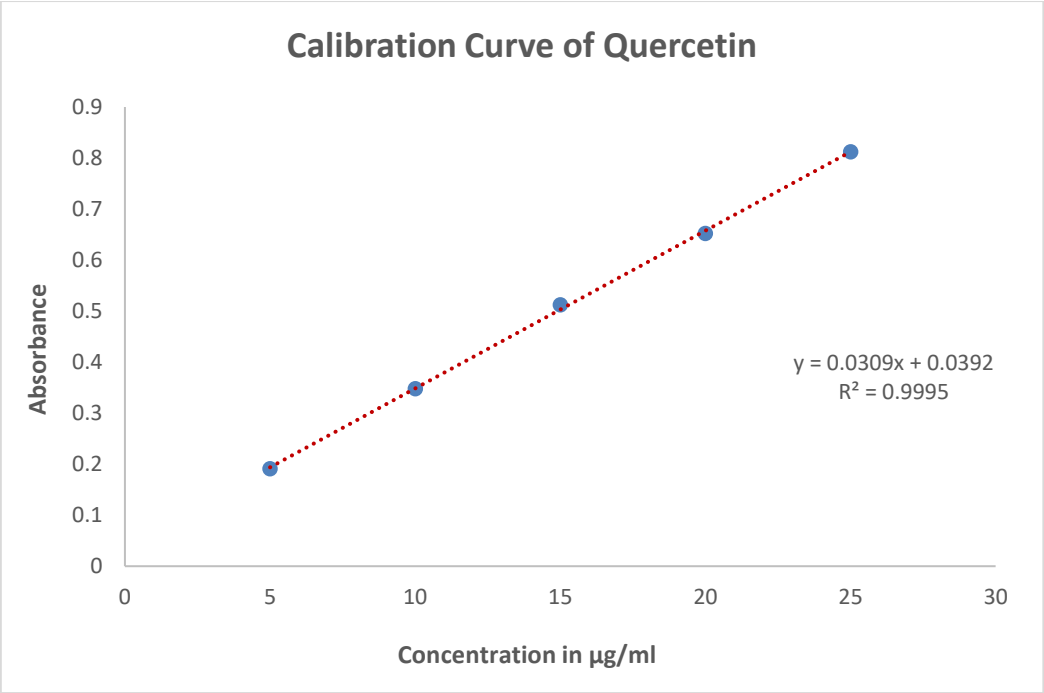
Graph No. 1: Calibration curve of Gallic acid

2.4.2 Total flavonoids content estimation (TFC) of *Excoecaria agallocha*

Using the equation based on the calibration curve: Y=0.032X + 0.018, R2=0.998, where X is the quercetin equivalent (QE) and Y is the absorbance, the total flavonoid content was estimated as quercetin equivalent (mg/100mg).

Table No. 5: Preparation of Calibration curve of Quercetin

S. No.	Concentration (µg/ml)	Absorbance
1	5	0.191
2	10	0.348
3	15	0.512
4	20	0.652
5	25	0.812
7	Hydoalcoholic extract of *Excoecaria agallocha*(10 µg/ml)	0.311



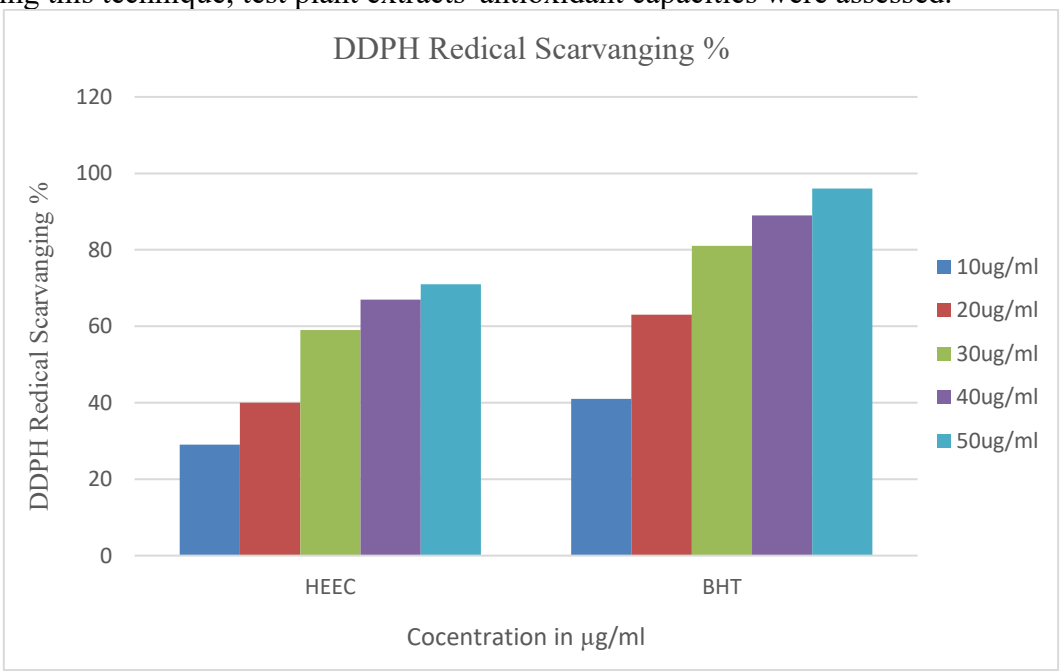
Graph No.2: Calibration Curve of Quercetin

Table No. 6: Estimation of total Phenol and flavonoids content of *Excoecaria agallocha* extracts

S. No.	Extract	Total phenol content (mg/ 100 mg of dried extract)	Total flavonoids content (mg/ 100 mg of dried extract)
1.	Hydoalcoholic extract of *Excoecaria agallocha*	35.22	7.99

2.4 DPPH radical Scavenging Abilities of the Test Plant Extracts

The highest absorption of the stable model free radical, DPPH, occurs at 515 nm and it has a rich purple colour. Antioxidant molecules have the ability to quench these radicals, which will then transform them into their reduced form (2,2-diphenyl-1-hydrazine), causing a reduction in absorbance at 515 nm (Ferreira et al., 2007). Utilizing this technique, test plant extracts' antioxidant capacities were assessed.

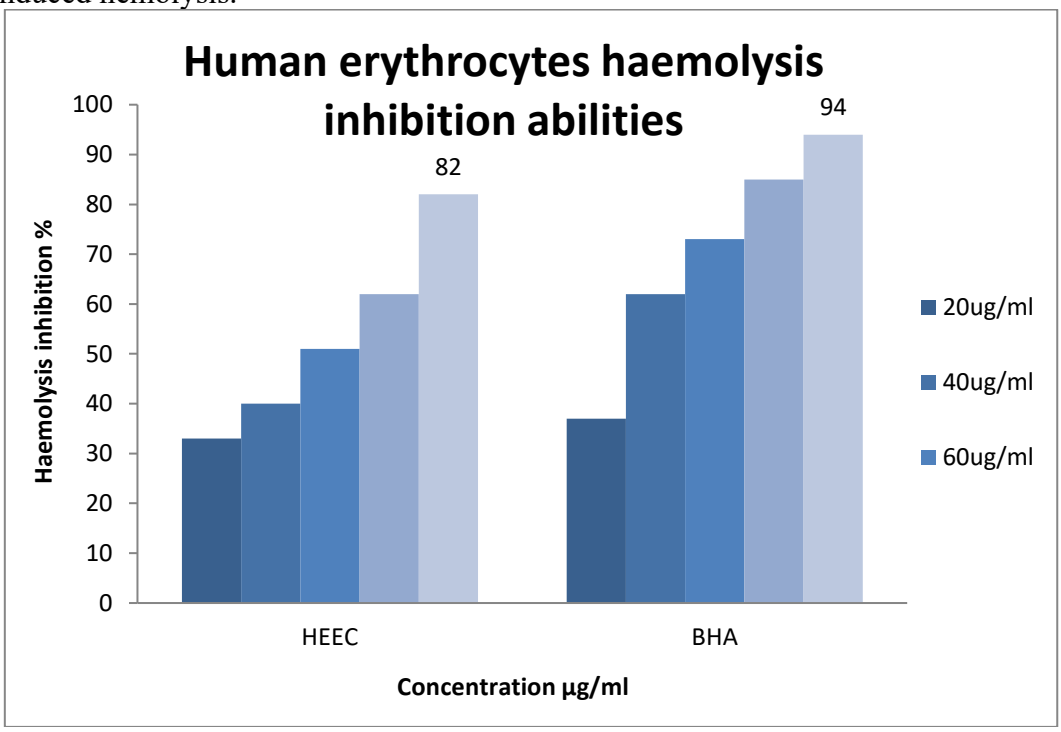


Graph No.3: DPPH Radical Scavenging Abilities of Both the extracts of *Excoecaria agallocha* compare to Standard

As shown in the graph (6.1 to 6.2), the test plants' capacity to scavenge DPPH radicals was ranked Excoecaria agallocha > Excoecaria agallocha. Among these plant extracts, the hydoalcoholic Excoecaria agallocha extract had the highest DPPH radical scavenging efficiency, 88% at a concentration of 50 g/ml. When compared to standard BHT, the hydoalcoholic extract of Excoecaria agallocha (71%) at a concentration of 50 g/ml demonstrated less radical scavenging ability.

2.5 Human erythrocytes haemolysis inhibition abilities of the test plant extracts

In the current study, erythrocytes were used to examine how well test plant extracts shielded the cells from free radical-induced hemolysis.



Graph No. 8: Haemolysis inhibition abilities of *Excoecaria agallocha* extracts

6.6 Detection and Calculation of R_f Value

1. The Rf Value of the spot was determined after the chromatogram was developed using the formula, and the results were shown in Table 1.

$$R_f = \frac{\text{Distance traveled by solute}}{\text{Distance traveled by solvent}}$$

6.6.1 Cyclophosphamide induced neutropenia

Cyclophosphamide (200 mg/kg, sc) administration led to a decrease in neutrophil count across all groups. However, compared to the control group, the neutrophil count decrease was less in the HEEC treated groups. While HEEC treatment caused a 44.80% decrease in TLC and a 30.27% decrease in neutrophil count and a 58% decrease in TLC in the control group. However, compared to the control

atment	Total leucocytes count (cells/mm3)		Reduction in cell number	% reduction	% neutrophil		Neutrophils reduction	% reduc
	Before	After			Before	After		
icle mL/kg,	5500.00±365.95	3600.00±333.33	3900.00±337.65	58.00	13.16±1.01	7.50±0.76	5.6±0.61	43.04

C kg, po)	5041.65±396.30	3783.33±359.70	358.33±315.80	44.80	13.65±1.30	8.83±1.35	**3.8±0.30***	30.37

All values are mean±SEM, n=5-6, *P<0.01 when compared to control group

6.6.2 Mice lethality test

HEEC (100 mg/kg, po) showed a and about 50% reduction in mortality, when compared to positive and negative controls.

Table No. 5: Effect in mice lethality test.

Treatment	Day 1	Day 3	Day 3	Mortality ratio
Negative control	1	5	-	100
Positive control + Vaccination	-	3	3	83.33
HEEC + Vaccination	-	3	1	50

Conclusion:

Therefore, it is possible to consider antioxidants as being vitally necessary for life in order to eradicate numerous pathological disorders and for overall welfare. Excoecaria agallocha extract may be important in this regard due to its known antioxidant qualities. With its bioactivity determination and active principle from Excoecaria agallocha, a powerful antioxidant and immunoprotective agent, the current investigation aims to uncover the depleting body of ethnomedical information about greens.

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