Preliminary Phytochemical Screening and *In Vitro* Antioxidant Activity of Methanolic Extract of *Aegle marmelos* (L) Correa Leaves

[1]Pinki Phougat, [1]Hitesh Kumar, [1]Praveen Nasa, [1]Neha Sharma

School of Pharmaceutical Sciences, Om Sterling Global University, Hisar

ABSTRACT

Natural products with medicinal value are gradually gaining importance in clinical research due to their well-known property of no side effects as compared to drugs. *Aegle marmelos* (L.) Correa (Rutaceae) commonly known as "bael" in Nepal and India, is a valuable medicinal plant and is considered sacred by the Hindus. It is used to cure several diseases in the Indian traditional medicine system of Ayurveda and has had similar uses among many ethnic communities residing in Indian subcontinent for over 5000 years. Its leaves, bark, stem, fruits and seeds have been used for various medicinal purposes. Recently the discovery of active components from the plant and their biological function in disease control has led to active interest in the plant across the globe. The aim of the present study was to evaluate physicochemical, qualitative and quantitative phytochemical analysis and *in vitro* antioxidant activities of leaves of *Aegle marmelos* collected from local areas of Sonipat, Haryana. The different physicochemical parameters were evaluated as per standard protocols with some modifications. Qualitative analysis of various phytochemical constituents and quantitative analysis of total phenolics and flavonoids were determined by the well-known test protocol available in the literature. Quantitative analysis of phenolic and flavonoids was carried out by Folins Ciocalteau reagent method and aluminium chloride method respectively. The *In vitro* antioxidant activity of methanolic extract of the leaves was assessed against DPPH and hydrogen peroxide scavenging assay method using standard protocols. The results obtained from the preliminary phytochemical screening of the methanolic extract of *Aegle marmelos* revealed the presence of carbohydrates, alkaloids, terpenoid, flavonoid, phenol, saponins and protein as major components. In petroleum ether extracts, only saponins are present. The total phenolics content of leaves methanolic extract was (84.5mg/100mg), followed

by flavonoids (69.5mg/100mg). The activities of methanolic leaves extract against DPPH and hydrogen peroxide scavenging assay method were concentration dependent. The diverse array of phytochemicals present in the plant thus suggests its therapeutic potentials which may be explored in drug manufacturing industry as well as in traditional medicine.

Keywords: *Aegle marmelos* (L.) Correa, Physicochemical, Phytochemical, Antioxidant, DPPH, H_2O_2

Introduction

India is a rich source of medicinal plants and a number of plant derived oils and extracts are used against diseases in various systems of medicine such as Ayurveda, Unani and Siddha. Only a few of them have been scientifically explored. Plant derived natural products such as phenols, flavonoids, terpenes and alkaloids [1, 2] have received considerable attention in recent years due to their diverse pharmacological properties. The qualitative analysis of phytochemicals of a medicinal plant is reported as vital step in any kind of medicinal plant research. Screening of plant constituents accurately can be done by employing chromatographic techniques [3]. Quantification usually employs the use of gravimetric and spectroscopic methods with several advanced approaches now available [4]. Reactive Oxygen Species (ROS), such as hydrogen peroxide, super oxide anion and hydroxyl radical, capable of causing damage to DNA, have been associated with carcinogenesis, coronary heart diseases and many other health problems related to advancing age [5]. Antioxidant that scavenges these free radicals proves to be beneficial for these disorders as they prevent damage against cell proteins, lipids and carbohydrates [6]. Erythrocytes, which are the most abundant cells in the human body, possessing desirable physiological and morphological characteristics are exploited extensively in drug delivery [7]. Oxidative damage to the erythrocyte membrane (lipid/ protein) may be implicated in haemolysis associated with some haemoglobinopathies, oxidative drugs, transition metal excess, radiation, and deficiencies in some erythrocyte antioxidant systems [8]. This assay is useful either for screening studies on various molecules and their metabolites, especially on one hand, molecule having an oxidizing or antioxidating activity or on the other hand, molecule having a long term action [9]. Several herbal secondary metabolites such as flavonoid have been found to protect cells from oxidative damage [10]. These compounds have been evidenced to stabilize RBC membrane by scavenging free radicals and reducing lipid peroxidation [11, 12]. Bael (*Aegle*

marmelos) has been known to be one of the most important medicinal plants of India since Charak (1500 B.C) [13]. More than 100 phytochemical compounds have been isolated from various parts of the plant, namely phenols, flavonoids, alkaloids, cardiac glycosides, saponins, terpenoids, steroids and tannins. These compounds are well known to possess biological and pharmacological activity against various chronic diseases such as cancer and cardiovascular and gastrointestinal disorders [14-17]. Antioxidant, antiulcer, antidiabetic, anticancer, antihyperlipidaemic, antiinflammatory, antimicrobial, antispermatogenic effects have also been reported on various animal models by the crude extracts of this plant [18]. Every part of *Aegle marmelos* plant such as its fruits, stem, bark, and leaves possesses medicinal property and is used for treating various eye and skin infections [19]. Leaf is considered to be one of the highest accumulatory parts of the plant containing bioactive compounds which are synthesized as secondary metabolites [20]. The aim of the present study was to evaluate physicochemical, qualitative and quantitative phytochemical analysis and *in vitro* antioxidant activities of leaves of *Aegle marmelos* collected from local areas of Sonipat, Haryana.

Materials and methods

Plant material

The leaves of *Aegle marmelos* were collected from local areas of Sonipat, Haryana. The identification and authentication of plant was done by Dr. Sunita Garg, Head, RHMD, CSIR-NIScPR, Delhi with reference authentication no. NIScPR/RHMD/Consult/2021/ 3896-97-2 dated 15/09/2021.

Chemical reagents

All the chemicals used in this study were obtained from Hi Media Laboratories Pvt. Ltd. (Mumbai, India), Sigma-Aldrich Chemical Co. (Milwaukee, WI, USA), SD Fine-Chem. Ltd. (Mumbai, India) and SRL Pvt. Ltd. (Mumbai, India). Clonidine (Unichem, Ltd.); Chlorpheniramine maleate (Alkem, Mumbai). All the chemicals used in this study were of analytical grade.

Soxhlet Extraction

Dried pulverized leaves of *Aegle marmelos* were placed in thimble of soxhlet apparatus. Soxhlation was performed at 60°C using petroleum ether (40-60ºC) as non-polar solvent at first. Exhausted plant material (marc) was dried and then extracted with methanol. For each solvent, soxhlation was continued till no colour was observed in siphon tube. For confirmation of

exhausted plant marc (i.e. completion of extraction), colorless solvent was collected from siphon tube and completion of extraction was confirmed by absence of any residual solvent. The entire extract was concentrated to dryness using rotary flash evaporator under reduced pressure and stored in an air tight container free from any contamination until it was used. Finally the percentage yields were calculated of the dried extracts [21].

Physicochemical parameters

Physicochemical parameters such as total ash value, alcohol soluble extractive value, water soluble extractive value and moisture content were determined using standard procedures [22, 23].

Phytochemical screening

Phytochemical screening to detect the presence of bioactive agents was performed by standard procedures [24, 25]. After the addition of specific reagents to the solution, the tests were detected by visual observation of color change or by precipitate formation.

Total phenolic content estimation

The amount of total phenolic in extracts was determined with the Folin Ciocalteu reagent. Concentrations of (20-100µg/ml) of gallic acid were prepared in methanol. Concentration of 100µg/ml of plant extract was also prepared in methanol and 0.5ml of each sample was introduced in to test and mixed with 2 ml of a 10 fold dilute folin Ciocalteu reagent and 4 ml of 7.5% sodium carbonate. The tubes were covered with parafilm and it was then incubated at room temperature for 30 minutes with intermittent shaking and the absorbance was taken at 765 nm against using methanol as blank. Total phenolic content was calculated by the standard regression curve of gallic acid and the results were expressed as gallic acid equivalent (mg/g) [26].

Total flavonoid content estimation

Different concentrations of rutin (20 to 100µg/ml) were prepared in methanol. Test sample of near about same polarity (100µg/ml) was prepared. An aliquot 0.5ml of diluted sample was mixed with 2 ml of distilled water and subsequently with 0.15 ml of a 5% $NaNO_2$ solution. After 6 minutes, 0.15 ml of a 10% $AlCl_3$ solution was added and allowed to stand for 5 minutes and then 2 ml of 4% NaOH solution was added to the mixture. The final volume was adjusted to 5ml with distilled water and allowed to stand for another 15 minutes. Absorbance was determined at

510 nm against water as blank. Total flavonoid content was calculated by the Standard regression curve of Rutin/ Quercetin [27].

***Invitro*-antioxidant activity**

DPPH radical scavenging activity

For DPPH assay, the method of Gulçin *et al.,* 2006 [28] was adopted. A solution of 0.1mM DPPH (4mg/100ml) in methanol was prepared and 1 ml of this solution was mixed with 1 ml of different concentrations of the different extracts. The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 min. Ascorbic acid was used as reference standard while methanol was used as control. Reduction of the stable DPPH radical was used as a marker of antioxidant capacity of *Aegle marmelos* extracts. The change in colour was measured at 517 nm wavelength using methanolic solution as a reference solution. This was related to the absorbance of the control without the plant extracts. The percentage inhibition of free radical DPPH was calculated from the following equation:

% inhibition = [(absorbance of control - absorbance of sample)/absorbance of control] × 100%.

All the tests were carried out in triplicates. Though the activity is expressed as 50% inhibitory concentration (IC50), IC50 was calculated based on the percentage of DPPH radicals scavenged. The lower the IC50 value, the higher is the antioxidant activity [29].

Hydrogen peroxide (H_2O_2) scavenging assay

Hydrogen peroxide scavenging activity of the plant extract was determined using the procedure explained by (Jayaprakasha *et al.,* 2004) [30]. A solution of hydrogen peroxide (20mM) was prepared in phosphate buffered saline (PBS; pH7.4). Hydrogen peroxide concentration was determined spectrophotometrically from absorption at 230nm by using the molar absorptivity of $81M^{-1}cm^{-1}$. Different concentrations of extract (20 to 100μg/ml) in ethanol were prepared. 1 ml of ethanolic standard and test were added to 2 ml of hydrogen peroxide solution in PBS. After 10 minutes the absorbance was measured at 230 nm against a blank solution that contained hydrogen peroxide solution without the extract [31].

The percentage of H_2O_2 scavenging of the plant extract was calculated as follows:

$$\%\ \text{scavenged}\ [\text{H2O2}] = \frac{(\text{Abs control} - \text{Abs sample})}{\text{Abs control}} \times 100$$

Results and discussion

The crude extracts so obtained after the soxhletion extraction process was further concentrated on water bath for evaporate the solvents completely to obtain the actual yield of extraction. The yield of *Aegle marmelos* petroleum ether and methanolic extracts was 0.13and 9.68% w/w respectively. Ash values and extractive values can be used as reliable aid for detecting adulteration. These studies help in identification of the plant materials. Ash values of drug also give an idea of earthy matter and other impurities present along with drug. Extractive values are primarily useful for the determination of exhausted and adulterated drugs. Extractive values are also useful to evaluate the chemical constituents present in the crude drug and help in estimation of specific constituents soluble in particular solvents. According to the given results, total ash value of *Aegle marmelos* was found to be 9.14, water and alcoholic extractive value were found to be 9.29 and 7.56, Moisture content of *Aegle marmelos* were also calculated, it was found to be 7.56.

The results of qualitative phytochemical analysis of the crude powder of flowers of *Aegle marmelos* are shown in Table 1. The results obtained from the preliminary phytochemical screening of the methanolic extract of *Aegle marmelos* revealed the presence of carbohydrates, alkaloids, terpenoid, flavonoid, phenol, saponin and protein as major components. In petroleum ether extracts, only saponins are present.

Table 1: Phytochemical evaluation of *Aegle marmelos* leaves

S. No.	Experiment	Result	
		Petroleum ether	Methanol
Test for Carbohydrates			
1.	Molisch's Test	-	+
2.	Fehling's Test	-	+
3.	Benedict's Test	-	+
4.	Bareford's Test	-	+
Test for Alkaloids			
1.	Mayer's Test	-	+
2.	Hager's Test	-	+
3.	Wagner's Test	-	+
4.	Dragendroff's Test	-	+
Test for Terpenoids			
1.	Salkowski Test	-	+

2.	Libermann-Burchard's Test	-	+
Test for Flavonoids			
1.	Lead Acetate Test	-	+
2.	Alkaline Reagent Test	-	+
3.	Shinoda Test	-	+
Test for Tannins and Phenolic Compounds			
1.	$FeCl_3$ Test	-	+
2.	Lead Acetate Test	-	+
3.	Gelatine Test	-	+
4.	Dilute Iodine Solution Test	-	+
Test for Saponins			
1.	Froth Test	+	+
Test for Protein and Amino acids			
1.	Ninhydrin Test	-	+
2.	Biuret's Test	-	+
3.	Million's Test	-	+
Test for Glycosides			
1.	Legal's Test	-	-
2.	Keller Killani Test	-	-
3.	Borntrager's Test	-	-

Quantitative phytochemical assay was performed by calculating total phenolic content (TPC) and total flavonoid content (TFC). The TPC was calculated with respect to gallic acid (standard) and TFC was then calculated with respect to rutin taken as standard. The TPC and TFC in methanolic extract were found to be 84.5mg/gm and 69.5mg/gm respectively (Table 2 & Figure 1, 2).

Table 2: Total phenolic and flavonoid content of extracts

Test	Methanolic extract
TPC	84.5mg/gm equivalent to Gallic acid
TFC	69.5 mg/gm equivalent to Rutin

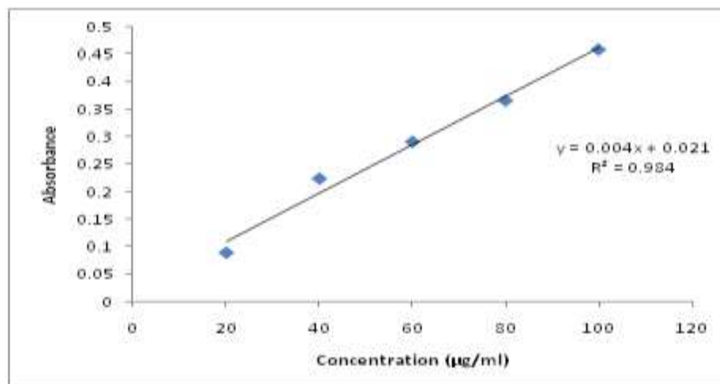

Figure 1: Graph of estimation of total phenolic content

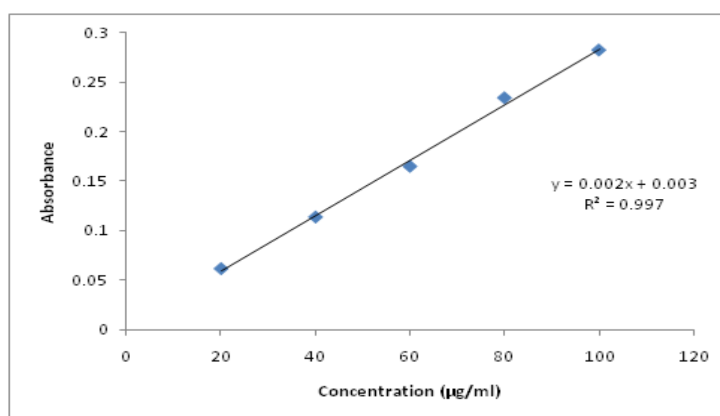

Figure 2: Graph of estimation of total flavonoids content

Antioxidant activity of the samples was calculated through DPPH and hydrogen peroxide scavenging assay method. % inhibition was calculated as an indicative of antioxidant potency. The higher the % inhibition, the better the activity. Ascorbic acid was taken as standard in all the 2 tests and the values were comparable with concentration ranging from 20µg/ml to100µg/ml. The reduction ability of DPPH radical was determined by the decrease in absorbance induced by plant antioxidants [32]. The methanolic extracts of *Aegle marmelos* observed a good inhibitory activity against DPPH radical. The scavenging activity of extracts and standard on the DPPH radical expressed as IC_{50} values: 47.39µg/ml and 10.92µg/ml as shown in Table 3. Highest quenching ability was shown by methanol extract.

Table 3: DPPH assay of ascorbic acid and methanolic extract

S. No.	Conc. (µg/ml)	Ascorbic acid (% Inhibition)	Methanolic Extract (% Inhibition)
1.	**20**	53.43	38.79
2.	**40**	61.24	48.17

3.	**60**	71.17	56.20
4.	**80**	79.51	62.46
5.	**100**	85.32	66.63
IC 50 Value		**10.92**	**47.39**

The antioxidant activity of plant extracts is due to polyphenols present in them which show redox properties. These are important since they decompose peroxides, neutralize free radicals, and quench singlet and triplet oxygen [33]. Table 4 shows the scavenging ability of methanolic extract and ascorbic acid on hydrogen peroxide at different concentrations.

Table 4: % Inhibition of ascorbic acid and methanolic extract using H_2O_2 method

S. No.	Conc. (µg/ml)	Ascorbic acid (% Inhibition)	Methanolic Extract (% Inhibition)
1.	**20**	51.98	35.87
2.	**40**	63.38	47.25
3.	**60**	69.39	54.75
4.	**80**	76.44	61.98
5.	**100**	85.78	72.15
IC 50 Value		**11.93**	**49.95**

Extracts are capable of scavenging hydrogen peroxide in an amount dependent manner at all the tested concentrations. Hydrogen peroxide itself is a rather weak oxidant and most organic compounds (except for some sulfur containing molecules) are virtually inert to attack by it at ordinary environmental or cellular concentrations and temperatures. In the presence of reduced transition metal ions, however, hydrogen peroxide is converted to the much more reactive oxidant, hydroxyl radical in the cells by Fenton reaction. Besides this, studies have shown that other transition metals such as copper (I), cobalt (II) and nickel (II) also take part in the process [34]. Thus, the removing is very important for antioxidant defense in cell or food systems. The scavenging activity of extracts and standard on the H_2O_2 radical expressed as IC_{50} values: 49.95µg/ml and 11.93µg/ml.

Conclusion

The plant under study can be considered a possible source of beneficial medications due to the presence of significant amounts of secondary metabolites in its leaves, including flavonoids, alkaloids, and phenolics. The fact that phytoconstituents are present in significant amounts may

help us to understand the potential pharmacological significance of this plant in the prevention and treatment of disease. The chemical components in plants that have distinct physiological effects on the human body are what give them their therapeutic worth. Additionally, it supports the statements made regarding the therapeutic benefits of this plant as a treatment method and its folkloric medical usage. Therefore, in order to produce viable chemotherapeutic drugs, we advise additional separation, purification, and characterization of the bioactive components from *Aegle marmelos* leaf, stem, flower, and seed.

References

1. Osawa T, Kawakishi S, Namiki M. Antimutagenesis and Anticarcinogenesis Mechanism II. New York.: Plenum Press; 1990. p. 139–53.
2. Keith MW, Sally AL, Michael WS, Thomas JG, Garry MM. Taxus spp. Needles contain amounts of taxol comparable to the stem bark of Taxus brevifolia: analysis and isolation. J Nat Prod. 1990;53(5):1249-55.
3. Sneader W. The discovery of aspirin: A reappraisal. Clin Res. 2000;321(7276):1591–4.
4. Aguoru CU, Ameh SJ, Olasan O. Comparative phyochemical studies on the presence and quantification of various bioactive compounds in the three major organs of okoho plant (cissus popunea guill & perr) in Benue State. Eur J Adv Res Biol Life Sci. 2014;2(2):16–22.
5. Beris H. Antioxidant affects a basis of drug selection. Drugs. 1991;42:569–605.
6. Marnett L. Oxyradicals and DNA damage. Carcinogenesis. 2000;21(3):361–70.
7. Hamidi H, Taferzadeh H. Carrier erythrocytes: an overview. Drug Del. 2003;10(1):9–20.
8. Ko F, Kuo F. Protection of oxidative haemolysis by dimethyl dineisoeugenol in normal and beta -thalassemia red blood cells. Free Radic Biol Med. 1997;22(1-2):215–22.
9. Djeridane A, Yous M, Nadjemi B, Vidal N, Lesgards J, Stocker P. Screening of some Algerian medicinal plants for the phenolic compounds and their antioxidant activity. European Food Res Technol. 2007;224:801–9.
10. Kumar S, Pandey AK. Chemistry and biological activities of flavonoids: An overview. Sci World J. 2013

11. Yu L. Free radical scavenging properties of conjugated linoleic acids. J Agric Food Chem. 2001;49(7):3452–546.
12. Ebrahimzadeh M, Nabavi S, Nabavi S, Eslami B. Antihemolytic and antioxidant activities of Allium paradoxum. Cent Eur J Biol. 2010;5(3):338–45.
13. Chemexcil, Selected Medicinal Plants of India, Basic Chemicals, Pharmaceutical and Cosmetic Export Promotion Council, Bombay, India, 1992.
14. L. Badam, S. S. Bedekar, K. B. Sonawane, and S. P. Joshi, “In vitro antiviral activity of bael (Aegle marmelos Corr.) upon human coxsackieviruses B1-B6,” Journal of Communicable Diseases, vol. 34, no. 2, pp. 88–99, 2002.
15. Y. L. Chew, J. K. Goh, and Y. Y. Lim, “Assessment of in vitro antioxidant capacity and polyphenolic composition of selected medicinal herbs from Leguminosae family in Peninsular Malaysia,” Food Chemistry, vol. 116, no. 1, pp. 13–18, 2009.
16. A. K. Gupta and N. Tandon, Reviews on Indian Medicinal Plants, vol. 1, Indian Council of Medicinal Research, New Delhi, India, 2004.
17. N. Kamalakkannan and P. Stanely Mainzen Prince, “Antihyperlipidaemic effect of Aegle marmelos fruit extract in streptozotocin-induced diabetes in rats,” Journal of the Science of Food and Agriculture, vol. 85, no. 4, pp. 569–573, 2005.
18. Mujeeb F, Bajpai P, Pathak N. Phytochemical evaluation, antimicrobial activity, and determination of bioactive components from leaves of Aegle marmelos. BioMed research international. 2014, Article ID 497606.
19. C. Kingston, S. Jeeva, G. M. Jeeva, S. Kiruba, B. P. Mishra, and D. Kannan, “Indigenous knowledge of using medicinal plants in treating skin diseases in Kanyakumari District, Southern India,” Indian Journal of Traditional Knowledge, vol. 8, no. 2, pp. 196– 200, 2009.
20. M. M. Cowan, “Plant products as antimicrobial agents,” Clinical Microbiology Reviews, vol. 12, no. 4, pp. 564–582, 1999.
21. Dutta R, Sharma MK, Jha M. Pharmacological evaluation of antiasthmatic activity of fumaria officinalis extracts. Plant Archives. 2020; 20(2):4308-15.
22. Dutta R, Sharma MK, Jha M. A Review on Ethnobotanical, Phytochemistry, Bioactivities and Medicinal Mysteries of Fumaria officinalis (Common Fumitory). EAS J. Pharm. Pharmacol. 2019; 1:99-105.

23. Ahmad T, Singh SB, Pandey S. Phytochemical screening and physicochemical parameters of crude drugs: A brief review. International Journal of Pharma Research & Review. 2013;2(12):53-60.
24. Jain DK, Gupta S, Jain R, Jain N. Anti-inflammatory activity of 80% ethanolic extracts of *Acorus calamus* Linn leaves in albino rats. Research Journal of Pharmacy and Technology. 2010; 3:882-4.
25. Jha M, Nema N, Shakya K, Ganesh N, Sharma V. In vitro hepatoprotective activity of Bauhinia variegata. Pharmacologyonline. 2009; 3:114-8.
26. Rani D, Jha M, Rai N, Kharkwal H. The in-vitro Sun Protection factor, estimation of Total Flavonoid, Phenol, Alkaloid Content of Leaf extracts of Grewia abutilifolia. Annals of the Romanian Society for Cell Biology. 2021;25(5):4249-59.
27. Joshi S, Parkhe G, Aqueel N, Dixit N, Jain DK. Estimation of total phenolic, total flavonoids and total protein content of hydroalcoholic extract of *Anacyclus pyrethrum.* Pharmacologyonline. 2019; 1:27-33.
28. Gulçin I, Elias R, Gepdiremen A, Boyer L. Antioxidant activity of lignans from fringe tree (Chionanthus virginicus L.). Eur Food Res Technol 2006; 223: 759-767.
29. Dutta R, Sharma MK, Khan A, Jha M. Phytochemical and in vitro antioxidant assay of Fumaria officinalis leaf extract. Journal of Advanced Scientific Research. 2020; 11(03):176-82.
30. Jayaprakasha GK, Rao LJ, Sakariah KK. Antioxidant activities of flavidin in different in vitro model systems. Bioorganic & Medicinal Chemistry 12 (2004) 5141-5146.
31. Karole S, Jain DK, Abbas S, Abbas N, Kare P. Antioxidant and hepatoprotective effects of *ougeinia dalbergioides* benth against paracetamol and ccl4 induced liver damage in rats. Indian Journal of Pharmacy and Pharmacology 2022; 9(1):67-74.
32. Rigane, G; Younes, S Ben; Ghazghazi, H; Salem, R Ben. Investigation into the biological activities and chemical composition of Calendula officinalis L. growing in Tunisia. International Food Research Journal; Selangor**.** 20(6), 2013: 3001-3007.
33. M. R. Saha, S. M. R. Hasan , R. Akter , M. M. Hossain , M. S. Alam , M. A. Alam, and M. E. H. Mazumder. In vitro free radical scavenging activity of methanol extract of the leaves of mimusops elengi linn. Bangl. J. Vet. Med. (2008). 6 (2): 197–202.

34. Halliwell, B.; Aeschbach, R.; Lolliger, J.; Aruoma, O. I. Food Chem. Toxicol. 1995, 33, 601.