Section A -Research paper

Physicochemical, Qualitative and Quantitative Determination of Secondary Metabolites and *in vitro* Antioxidant Potential of *Bombax ceiba* Linn Flower Extracts

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

School of Pharmaceutical Sciences, Om Sterling Global University, Hisar

ABSTRACT

Bombax ceiba Linn (Bombacaceae) is a tall tree buttressed at the base that is widely distributed throughout India, Ceylon and Malaya up to 1500 m of altitude. Many parts of the plant (root, stem bark, gum, leaf, prickles, flower, fruit, seed and heartwood) are used by various tribal communities and forest dwellers for the treatment of a variety of ailments. It also possesses important pharmacological activity such as aphrodisiac, anti-inflammatory and hepatoprotective activity in addition to anticancer and anti-HIV activity, anti-Helicobacter pylori, antiangiogenic, analgesic and antioxidant activity and hypotensive, hypoglycemic and antimicrobial activity. It is reported to contain important phytoconstituents such as naphthol, naphthoquinones, polysaccharides, anthocyanins, shamimin and lupeol. The aim of the present study was to evaluate physicochemical, qualitative and quantitative phytochemical analysis and *in vitro* antioxidant activities of flower of *Bombax ceiba* 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 flowers was assessed against DPPH and hydrogen peroxide scavenging assay method using standard protocols. Phytochemical estimation of petroleum ether extract of *Bombax ceiba* showed the presence of terpenoids and saponins. Flavonoids, alkaloids, carbohydrates, tannins and phenolic compounds, saponins, protein and

amino acids, glycosides were detected in methanolic extractof *Bombax ceiba*. The total phenolics content of flowers methanolic extract was 45.75mg/100mg, followed by flavonoids 38.5mg/100mg. The activities of methanolic flower 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: *Bombax ceiba* Linn, Physicochemical, Phytochemical, Antioxidant, DPPH, H_2O_2

Introduction

There has been intense interest recently among the public and the media in the possibility that increased intake of dietary antioxidants may protect against chronic diseases, which include cancers, cardiovascular, and cerebrovascular diseases. Antioxidants are substances that, when present at low concentrations, compared with those of an oxidizable substrate, significantly prevent or delay a pro-oxidant-initiated oxidation of the substrate [1]. A pro-oxidant is a toxic substance that can cause oxidative damage to lipids, proteins, and nucleic acids, resulting in various pathological events or diseases. Examples of pro-oxidants include reactive oxygen and nitrogen species (ROS and RNS), which are products of normal aerobic metabolic processes. ROS include superoxide ($O_2-\cdot$), hydroxyl ($OH\cdot$), and peroxyl ($ROO\cdot$) radicals, and hydrogen peroxide (H_2O_2). RNS include nitric oxide ($NO\cdot$) and nitrogen dioxide ($NO_2\cdot$) [2,3]. There is considerable biological evidence that ROS and RNS can be damaging to cells and, thereby, they might contribute to cellular dysfunction and diseases. The existence and development of cells in an oxygen-containing environment would not be possible without the presence of a complicated antioxidant defense system that includes enzymatic and nonenzymatic components. The nonenzymatic antioxidants, most of which have low molecular weights and are able to directly and efficiently quench ROS and RNS, constitute an important aspect of the body's antioxidant system components [4]. The interaction among these antioxidants and the difficulty in measuring all of them individually prompted the development of assays for measuring total antioxidant capacity. The measurement of total antioxidant capacity of all these nonenzymatic antioxidants is necessary and important in evaluating in vivo antioxidant status in many clinical and nutritional studies. During the last decade, there was a growing demand for natural plants having diverse activities towards diseases especially chronic ones that need long term management [5]. *Bombax ceiba* Linn belongs to the family Bombacaceae and is a vital medicinal plant cultivated

throughout tropical and subtropical Asia. The plant is well known among the tribal people for the treatment of various diseases related to humans as well as animals. According to Ayurveda, it possesses anti-dysenteric, astringent, diuretic, stimulant, haemostatic, anti-diarrheal, cardiotonic, demulcent and antipyretic effects [6]. Traditionally, different parts of *Bombax ceiba* are being used for the treatment of various disorders. The roots of the plant are used for the treatment of wounds, diarrhea and dysentery while the gum is useful in burning sensation, pulmonary tuberculosis, enteritis and influenza. The flowers are good for skin problems and bark is demulcent and emetic. The fruits are useful in chronic inflammation and ulceration of kidney and bladder while the seeds are good in treating gonorrhoea. The literature reports reveal its antioxidative, anti-inflammatory, antihyperglycemic, antihyperlipidemic, immunomodulatory, and hepatoprotective activity [7-10]. Different classes of compounds present in *Bombax ceiba* leaves are alkaloids, flavanoids, carbohydrates, quinones, cardiac glycoside, saponins, phenols, tannins and terpenoids [11]. The polysaccharide fraction of *Bombax ceiba* flower is reported to have immunomodulatory activity [12]. The aim of this work was to determine the quality (types), quantity (amount) of bioactive compounds and in vitro antioxidant activity of flowers of *Bombax ceiba* in local areas of Sonipat, Haryana.

Materials and methods

Plant material

The flowers of Bombax *ceiba* 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/2021/3896-97-3 dated 15/09/2021.

Chemical reagents

All the chemicals used in this study were obtained from HiMedia 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 flowers of *Bombax ceiba* 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 [13].

Physicochemical parameters

Total ash value

A silica dish was used to burn 5 gm of powdered medication at a temperature no higher than 450◦C until it was carbon free in a muffle furnace. After cooling, it was weighed. It was determined what percentage of the drug's weight was ash.

Alcohol soluble extractive value

In a closed flask, 5 gm of coarsely powdered, air-dried medication was macerated for 24 hours with 100 ml of alcohol, shaking frequently for the first six hours, and then left to stand for the last 18 hours. After that, it was quickly filtered to prevent alcohol loss. A 25 ml sample of the filtrate was dried to dryness in a shallow dish with a flat bottom, dried at 105 ^{o}C, and weighed. The percentage of extractive that is soluble in alcohol was estimated using the air-dried medication as a base.

Water soluble extractive value

A closed flask was used to macerate 5gm of coarsely powdered, air-dried medication with 100 ml of chloroform water for 24 hours, stirring frequently for six hours, then allowing to stand for eighteen hours. Following that, it was quickly filtered while taking measures to prevent chloroform water loss. A 25ml sample of the filtrate was dried to dryness in a flat-bottomed dish that had been dried at 105 ^{o}C before being weighed.

Moisture content

Place 2 to 6 g of the sample into a weighing bottle which has been accurately weighed, and weigh it accurately. Then, dry it at 105 ^{o}C for 5 - 6 hours and cool it in desiccators with silica gel. When the material is dried to a constant weight, the moisture content is determined [14].

Phytochemical screening

Phytochemical screening to detect the presence of bioactive agents was performed by standard procedures [15, 16]. 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. Concentration of (20-100µg/ml) of gallic acid was prepared in methanol. Concentration of 100µg/ml of plant extract were also prepared in methanol and 0.5ml of each sample were 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 min with intermittent shaking and the absorbance were 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) [17].

Total flavonoid content estimation

Different concentration of rutin (20 to 100µg/ml) was prepared in methanol. Test sample of near about same polarity (100µg/ml) were 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 min, 0.15 ml of a 10% $AlCl_3$ solution was added and allowed to stand for 5min, 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 min. Absorbance was determined at 510 nm against water as blank. Total flavonoid content was calculated by the Standard regression curve of Rutin/ Quercetin [18].

***Invitro*-antioxidant activity**

DPPH radical scavenging activity

For DPPH assay, the method of Gulçin *et al.,* 2006 [19] 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 *Bombax ceiba* 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 [20].

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) [21]. 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 [22].

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

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

Results and discussion

The crude extracts so obtained after the soxhletion extraction process was further concentrated on water bath to evaporate the solvents completely to obtain the actual yield of extraction. The yield of *Bombax ceiba* petroleum ether and methanolic extracts was 0.22 and 2.43% 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 *Bombax ceiba* was found to be 4.61, Water and alcoholic extractive value were found to

be 8.48 and 7.62, Moisture content of *Bombax ceiba* was also calculated, it was found to be 6.89. The results of qualitative phytochemical analysis of the crude powder of flowers of *Bombax ceiba* are shown in Table 1. Phytochemical estimation of petroleum ether extract of *Bombax ceiba*, showed the presence of terpenoids, and saponins. Flavonoids, alkaloids, carbohydrates, tannins and phenolic compounds, saponins, protein and amino acids, glycosides were detected in methanolic extract of *Bombax ceiba*.

Table 1: Phytochemical evaluation of *Bombax ceiba* flowers

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 45.75mg/gm and 38.5mg/gm respectively (Table 2 & Figure 1, 2).

Table 2: Total phenolic and flavonoid content of extracts

Test	Methanolic extract
TPC	45.75 mg/gm equivalent to Gallic acid
TFC	38.5 mg/gm equivalent to Rutin

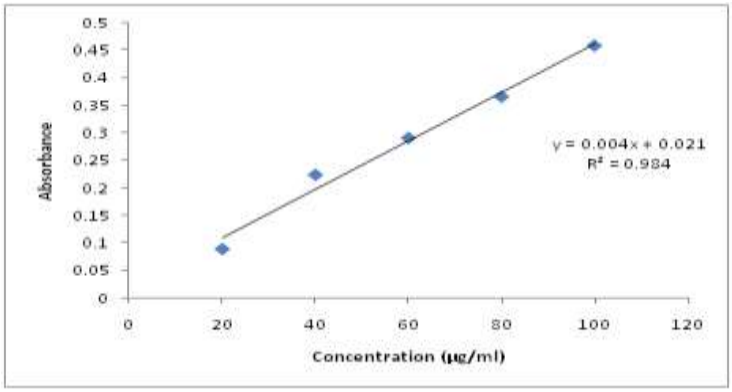

Figure 1: Graph of estimation of total phenolic content

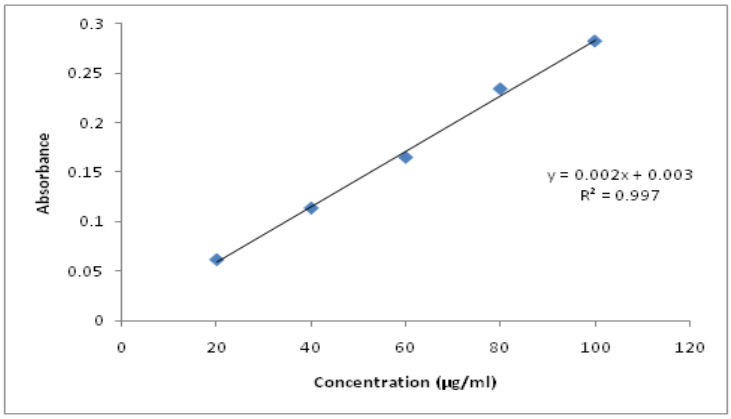

Figure 2: Graph of estimation of total flavonoids content

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 to

100µg/ml. The reduction ability of DPPH radical was determined by the decrease in absorbance induced by plant antioxidants [23]. The methanolic extracts of *Bombax ceiba* observed a good inhibitory activity against DPPH radical. The scavenging activity of extracts and standard on the DPPH radical expressed as IC_{50} values: 51.879µg/ml and 10.92µg/ml (Table 3).

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	37.22
2.	**40**	61.24	45.35
3.	**60**	71.17	52.97
4.	**80**	79.51	61.83
5.	**100**	85.32	68.92
IC 50 Value		**10.92**	**51.879**

Highest quenching ability was shown by methanol extract. 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 [24]. Table 4 shows the scavenging ability of methanolic extract and ascorbic acid on hydrogen peroxide at different concentrations. Extracts were capable of scavenging hydrogen peroxide in an amount dependent manner at all the tested 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	32.93
2.	**40**	63.38	38.28
3.	**60**	69.39	56.09
4.	**80**	76.44	65.99
5.	**100**	85.78	70.95
IC 50 Value		**11.93**	**54.59**

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 [25]. 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: 54.59µg/ml and 11.93µg/ml.

Conclusion

It can be concluded from present investigation that the observed level of phytoconstituents revealed that *Bombax ceiba* is a rich source of antioxidant compounds proved by in vitro studies. Currently available synthetic antioxidants are suspected to cause or prompt negative health effects, hence strong restrictions have been placed on their application and there is a trend to substitute them with naturally occurring antioxidants. The broad range of activity of the extracts suggests that multiple mechanisms are responsible for the antioxidant activity. The multiple antioxidant activity of extract demonstrated in this study clearly indicates the potential application value of the *Bombax ceiba*. However, the in vivo safety of *Bombax ceiba* needs to be thoroughly investigated in experimental rodent models prior to its possible application as an antioxidant ingredient, either in animal feeds or in human health foods. Further studies, on the use of above plants for their antioxidant role in various systems may provide potential natural antioxidants.

References

1. Prior RL, Cao G (1999) In vivo total antioxidant capacity: comparison of different analytical methods. Free Rad Biol Med 27:1173–1181.
2. Gulçin İ (2012) Antioxidant activity of food constituents: an overview. Arch Toxicol 86:345–391.
3. Gulçin İ, Topal F, Çakmakçı R, Bilsel M, Gören AC, Erdogan U (2011) Pomological features, nutritional quality, polyphenol content analysis, and antioxidant properties of domesticated and 3 wild ecotype forms of raspberries (Rubusidaeus L.). J Food Sci 76:C585–C593.
4. Cao G, Prior RL (2002) Hand book of Antioxidants. In: Cadenas E, Packer L (ed) Measurement of total antioxidant capacity in nutritional and clinical studies, CRC Press, 2nd edition. Marcel Dekker Inc, New York, pp 1–2.

5. Raafat K. The top 100 phytoremedies and caregiving. 1st ed. Beirut: Dar Al-Nahda Al-Arabia; 2016.
6. Meena V, Chaudhary AK. Shalmali (Bombax ceiba): Versatility in its therapeutics. Int J Green Pharm 2017; 11(3): 401-406.
7. Bora MM, Deka C, Tapadar SA, Jha DK, Kakati DK. Alkyds from red silk-cotton (Bombax ceiba) seed oil and investigation on their microbial degradation. Progr Org Coat 2018; 124: 71-79.
8. Xu GK, Qi XY, Wa GK, Xi GY, Li XS, Su CY, et al. Antihyperglycemic, antihyperlipidemic and antioxidant effects of standard ethanol extract of Bombax ceiba leaves in high-fat-diet-and streptozotocin-induced type 2 diabetic rats. Chin J Nat Med 2017; 15(3): 168-177.
9. Arafa AF, Foda DS, Mahmoud AH, Metwally NS, Farrag ARH. Bombax ceiba flowers extract ameliorates hepatosteatosis induced by ethanol and relatively moderate fat diet in rats. Toxicol Rep 2019; 6: 401-408.
10. Zhang YB, Wu P, Zhang XL, Xia C, Li GQ, Ye WC, et al. Phenolic compounds from the flowers of Bombax malabaricum and their antioxidant and antiviral activities. Molecules 2015; 20(11): 19947-19957.
11. Faizi S, Zikr-ur-Rehman S, Naz A, Versiani MA, Dar A, Naqvi S. Bioassay-guided studies on Bombax ceiba leaf extract: Isolation of shamimoside, a new antioxidant xanthone C-glucoside. Chem Nat Comp 2012; 48(5): 774-779.
12. Hait M, Goswami J. Physicochemical and phytochemical status on flower of Bombax ceiba. J Med Plants 2017; 5(3): 189-192.
13. Dutta R, Sharma MK, Jha M. Pharmacological evaluation of antiasthmatic activity of fumaria officinalis extracts. Plant Archives. 2020; 20(2):4308-15.
14. 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.
15. 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.

16. Jha M, Nema N, Shakya K, Ganesh N, Sharma V. In vitro hepatoprotective activity of Bauhinia variegata. Pharmacologyonline. 2009; 3:114-8.
17. 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 Grewiaabutilifolia. Annals of the Romanian Society for Cell Biology. 2021;25(5):4249-59.
18. 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.
19. Gulçin I, Elias R, Gepdiremen A, Boyer L. Antioxidant activity of lignans from fringe tree (ChionanthusvirginicusL.). Eur Food Res Technol2006; 223: 759-767.
20. 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.
21. Jayaprakasha GK, Rao LJ, Sakariah KK. Antioxidant activities of flavidin in different in vitro model systems. Bioorganic & Medicinal Chemistry 12 (2004) 5141-5146.
22. Karole S, Jain DK, Abbas S, Abbas N, Kare P. Antioxidant and hepatoprotective effects of *ougeiniadalbergioides*benth against paracetamol and ccl4 induced liver damage in rats. Indian Journal of Pharmacy and Pharmacology 2022; 9(1):67-74.
23. 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.
24. 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 mimusopselengilinn. Bangl. J. Vet. Med. (2008). 6 (2): 197–202.
25. Halliwell, B.; Aeschbach, R.; Lolliger, J.; Aruoma, O. I. Food Chem. Toxicol. 1995, 33, 601.