

Phytochemical screening and *in vitro* antioxidant activity analysis of leaf and callus extracts of *Allophylus serratus* (ROXB) KURZ.

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ABSTRACT

Allophylus serratus is an important medicinal plant whose leaves, fruits, roots and stem have been in use in traditional medicines. The present study is aimed to carry out preliminary phytochemical analysis and to determine antioxidant activity of leaf and callus water, methanol, ethyl acetate, chloroform and petroleum ether extracts of *A. serratus*. The extracts were subject to various qualitative and quantitative tests for phytochemical analysis. An *in vitro* antioxidant activity of the extracts was investigated by DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS⁺⁺, (2,2-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid) and FRAPS (Ferric reducing/ antioxidant power) assays. The qualitative phytochemical analysis confirmed the presence of alkaloids, phenols, flavonoids, glycosides, saponins, tannins, and steroids in both leaf and callus extracts. The quantitative analysis result showed higher yields of phenolics (104.73±4.3 mg) followed by flavonoids (69.63±6.35 mg) and soluble tannins (69.59±1.27mg) equivalent gallic acid/g of methanol, ethyl acetate and methanol, respectively. In case of callus extracts, the highest yield is phenols (54.42±6.59gm) followed by flavonoids (39.17±6.35 gm) equivalent gallic acid/g of methanol extracts. The antioxidant activity assays of both leaf and callus extracts demonstrated strong antioxidant activity which could be due to high content of phenolics and flavonoids. The methanol extract showed more antioxidant potential than the other extracts of both leaf and callus but leaf extracts show significant antioxidant activity than the callus extracts. Therefore, both leaf and callus extracts of *Allophylus serratus* contain phytochemicals that are potential source for antioxidant which suggested that the plant could serve as a source of useful drugs.

Keywords: *Allophylus serratus*, antioxidant activity, phytochemical screening, callus extract.

1. INTRODUCTION

Plants have been used all over the world as a source of traditional and modern medicine. They have made huge contributions to human health and wellbeing by providing lifesaving drugs for the majority of the world population. In traditional medicine which covers over 80% of the world's population, medicinal plants play important role in the treatment of many ailments, especially in the

developing world (1).

The medicinal properties of medicinal plants are mainly due to the presence of different complex phytochemical constituents which are found as secondary metabolites. The most important of these bioactive phytochemical constituents of plants are alkaloids, glycosides, steroids, saponins, tannins, anthraquinones, flavonoids and phenolic compounds which produce physiological action on the human body (2). The phytochemicals that plants produce in their various parts such as leaves, barks, flowers, roots, fruits and seeds have various applications. For example, phenolic, one of the

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largest and most common group of plant secondary metabolites (3,4) are used as antioxidants and free radical scavengers (5,6). Other phytochemicals produced by medicinal plants have also been used as phyto-medicines (7). Knowledge of plants phytochemical constituent is very important to discover new therapeutic agents from plants and to disclose the new source of economically important materials such as oils, gums, tannins, saponins etc. which are precursors for the synthesis of complex chemical products (8,9).

Many research reports revealed that medicinal plants have the ability to cure diseases and improve human health without side effects. At present interests in herbal medicine are increasing globally and medicinal plants are also attracting the attention of drug and cosmetic industries due to two important reasons. First, there is an increase in drug resistance of microorganisms to synthetic drugs and drug allergies which indicates that there is intolerance to chemically synthesized medicines and presence of side effects of these chemical drugs. Second, increase in international pharmaceutical drug industries interest to use a traditional indigenous treatment as drug sources or to discover new drugs (10). According to (11) approximately two-thirds of drugs approved worldwide are predicted to be derived from plants. Thus, to discover new sources of alternative medicines, many types of research have been carried out on medicinal plants (12). The knowledge of the phytochemical compounds present in medicinal plants help us in designing new, safe and effective formulations of phyto-therapeutics and nutraceuticals for humans.

A. serratus, from family Spandaceae, is a semi evergreen large shrub or small tree which has astringent, bitter, sweet, anti-inflammatory, vulnerary, digestive, carminative and constipating properties. Traditionally it is used for the treatment of elephantiasis, edema, fractured bones, dislocations, inflammations, ulcers, wounds, and several gastrointestinal disorders such as dyspepsia, anorexia, and diarrhea (13). This plant is also used as a carminative drug and reducing blood glucose(14). Almost

all parts of *A. serratus* plant have medicinal importance and used traditionally for the treatment of various ailments such as reduce fever, to relieve rashes, promote lactation, to treat colic, to relieve stomach aches, asantiulcer (15,16).

Several previous studies have demonstrated that different parts of this pant have pharmacological activities such as anti-inflammatory, anti-osteoporotic and anti-ulcer, antiviral and anti-bacterial (17). Different phytochemicals present in *A. serratus* are responsible for these pharmacological activities. Thus the preliminary phytochemical screening of plants and antioxidant activity test is very important in identifying new sources of therapeutically important compounds like flavonoids, alkaloids, phenolic compounds, saponins, tannins, steroids, terpenoids etc. It is enviable to know the phytochemical composition and amount of phytochemicals in the plant material before testing its value for medicinal purpose. Therefore, the present study was aimed at the preliminary phytochemical screening, estimation of the amount of some phytochemicals and testing the antioxidant activities of leaf and callus extracts of *A. serratus*.

2. Materials and Methods

2.1. Callus Induction and Production

The leaves collected from the field were used as explants for callus induction. The leaves of *A. serratus* were collected from Andhra University Campus, Visakhapatanam and authenticated at the Department of Botany, Andhra University, India, and a voucher specimen numbered (21921) were deposited in the Herbarium, College of Science and Technology, Department of Botany, Andhra University, India.

The explants were cut into 1.5 -.5 cm and thoroughly washed under running tap water and local detergent (Dettol). The explants were then surface sterilized with 70% ethanol for 1 minute followed by 0.1% HgCl₂ for 2minutes and 1% NaOCl for 3minutes. For callus induction and proliferation MS (18) medium supplemented with 3mg/IBAP and 0.5mg/l NAA were used. MS medium without any plant growth regulators was used as a control. The explants were cut into required

sizes (1.0-2.0 cm) and inoculated onto MS medium fortified with 3 mg/l BAP and 0.5mg/l NAA to initiate the callus phase. The callus for this study were obtained from the 60 days age of leaves callus derived from the same plant and cultured on MS media (Murashige, and F Skoog 1962) supplemented with 3mg/l BAP and 0.5mg/l NAA.

2.2. Preparation of leaf and callus extracts

The leaves and leaf derived callus of *A.serratus* were washed with distilled water and air dried in dark for three weeks to a constant weight. The dried samples were then ground into powder using a clean electric blender. The powdered materials were kept in air tight container and protected from sunlight until used. 100 grams of the leaf and leaf derived callus powders were extracted using Soxhelt extractor in different solvents such as water, methanol, ethyl acetate, chloroform and petroleum ether separately. The extracts were concentrated and vacuum dried on rotary evaporator and only crude extracts were left. The percentage yield (weight) of each solvent extracts were calculated. The extracts were kept at 4°C until used for qualitative, quantitative phytochemical analysis and antioxidant activity assessment.

2.3. Qualitative phytochemical Screening

The leaf and leaf derived callus extracts of were subject to different preliminary phytochemical tests to establish the profile of extracts for the presence of important phytochemicals. Chemical tests were carried using standard procedures for the detection of alkaloids (Mayer's and Wagner's test), phenols and tannins (Ferric chloride test and Lead acetate test) flavonoids (Lead acetate test and ammonium solution tests), saponins (foaming index), steroids ((Salkowski's test Liebermann-Buchard reaction), terpenoids (Salkowski's test and cardiac glycosides (Liebermann's test) in order to identify the presence of phytochemical constituents (19-21).

2.4. Quantitative phytochemical analysis

The determination of the total content of some detected phytochemicals such as alkaloids, phenolics, flavonoids, saponins and tannins was carried out according to

previously described standard methods as follows.

2.4.1. Determination of total alkaloids content

Total Alkaloid determination was done by using (22) method. One gram of dry samples of the leaf and callus were separately placed in a 250ml beaker and 100ml of 10% acetic acid in ethanol was added. The mixtures were covered and allowed to stand for 4 hours and then filtered through WhatmanNo1 filter paper. The filtrates were concentrated on a hot water bath until the volume reaches up to 25ml. To these concentrated solutions; concentrated ammonium hydroxide (NH₄OH) was added drop wise until precipitation formed completely. The mixtures were then allowed to stand for 5 minutes and filtered through a weighed filter paper. The precipitates collected on a weighed filter paper were washed with dilute ammonium hydroxide (NH₄OH). The residue on the filter paper was alkaloid, which was then dried and weighed. The percentages of alkaloid were calculated by the difference.

2.4.2. Determination of total phenols

The total phenolic content was determined according to Folin-Ciocalteu reagent procedure (23) with some modifications. In brief, 100 µl of the leaf and callus extracts (1mg/ml) were taken separately in test tubes and distilled water was added to make the volume 1.0 ml. Then 1.0 ml of Folin-Ciocalteu reagent (1:1 with water) and 0.8 ml of sodium carbonate (Na₂CO₃) solution (10%) were added sequentially in each tube and the mixtures were vortexed. Then the test tubes were incubated in dark for 2 hours at room temperature. Gallic acid at concentrations 0- 400 mg/l was used as a standard to plot the calibration curve. The absorbances of the mixtures were recorded at 725 nm against the reagent blank using -UV-Vis spectrophotometer. The analysis was performed in triplicate and the results were expressed as percentage milligram gallic acid equivalents (GAE) per gram of the dry weight of extract as calculated from gallic acid standard graph.

2.4.3. Determination of total flavonoid content

The quantification of flavonoid content of leaf and leaf derived callus extracts was determined by Aluminium chloride

(AlCl₃) calorimetric assay method (24) with slightly modification. One ml of the appropriately diluted leaf and callus extracts (1 mg/ml) solutions were separately prepared and mixed with 1 ml of distilled water and subsequently with 75 µl of 5 % NaNO₂ solution. After 5 min, 75 µl of 10% AlCl₃H₂O solution was added and allowed to stand for 5min, and then 0.5 ml of 1MNaOH solution was added to the mixture. The mixtures were thoroughly mixed by adding water to bring the final volume to 5 ml, and then allowed to stand for 30min at room temperature. The increase in absorbance of the mixtures was determined at 510 nm versus water as a blank. Gallic acid was used as a standard in concentrations of 0- 400 mg/l was used to make the calibration curve. The total flavonoid contents were calculated using standard Gallic acid calibration curve. The analysis was performed in triplicate and the results were expressed as percentage milligrams of Gallic acid equivalent (GAE) per gram of the dry weight of extracts (25).

2.4.4. Determination of total tannins

Determination of total content of tannins was done according to previously described Folin–Ciocalteu method using spectrophotometer (26). Briefly, about 0.1 ml of the leaf and callus extracts (1mg/ml) were added to a volumetric flask (10 ml) and 6.6 ml of distilled water was added. Then 0.5 ml of Folin-Ciocalteu phenol reagent and 1.5 ml of 20 % Na₂CO₃solution were added sequentially and dilute to 10 ml with distilled water. The mixture was shaken well and kept at room temperature for 30 min. gallic acid at concentrations of 0-400 mg/l were prepared. Absorbances for standard and test solutions were measured against the blank at 725 nm with a UV/Visible spectrophotometer. The analysis was performed in triplicate, and the tannins content was expressed as milligram gallic acid equivalents (GAE) per gram of the dry weight of extracts as calculated from the tannic acid standard graph.

2.4.5. Determination of Saponins

Total saponin content determination was carried out according to the method of (27). In brief, 10 g of powder of the samples were put into a conical flask (250ml) and 100 ml of 20% Ethanol (C₂H₅OH) was added to them. The samples were

heated over a hot water bath at 60°C for 3 hours with continuous stirring. The mixtures were then filtered and the supernatants were separated from the residues. The solid residues were re-extracted with another 200 ml of 20% ethanol. The solutions were filtered and the extracts were mixed with the previous extracts separately. The combined extracts were put on water bath at about 90°C and heated till the volume of the extracts was reduced to 40 ml. The concentrated extracts were transferred into a 250 ml separator funnel and 10 ml of diethyl ether (CH₃CH₂)₂O was added to the extracts and shaken vigorously. After the solution settle down, the aqueous layers were recovered while the diethyl ether (CH₃CH₂)₂O layers were discarded. The purification processes were repeated. Then 60 ml of n-butanol (n-C₄H₉OH) was added and the combined n-butanol (n-C₄H₉OH) extracts were washed twice with 10 ml of 5% aqueous Sodium chloride (NaCl)solution. The remaining solutions were then heated in a water bath at about 50 °c until the solvent evaporates. After the solvent evaporated, the extracts were then dried in an oven to a constant weight. The total saponin contents were calculated by the following equation:

$$\text{Saponin content} = \frac{\text{WEP}}{\text{WS}} \times 100$$

Where, WEP = Weight of oven dried end products.
WS= Weight of powdered samples taken for test.

2.5. Antioxidant Activity Assessment

2.5.1. Evaluation of antioxidant activity by DPPH radical scavenging method

Free radical scavenging activity of extracts of leaves and callus were measured by DPPH according to the method described previously (28 and 29) with slight modification. In brief, 1ml of 0.1 mM solution of DPPH in methanol solution was mixed with 1ml of aqueous and methanolic extracts of leaves and callus at different concentration (100, 200, 400, 600, 800, 1000 µg/ml). Ascorbic acid was used as reference standard compound and positive control whereas methanol served as a blank. The mixtures were vortexed and incubated at room temp

for 30 minutes. The experiments were done in triplicate and the decrease in absorbance was measured at 517 nm by using spectrophotometer (UV-VIS Shimadzu). The DPPH scavenging activity percent (%) was calculated by using following formula:

$$\text{Percent inhibition (\%)} = \frac{A_{bc} - A_{bs}}{A_{bc}} \times 100$$

Where A_{bc} is the Absorbance of control (DPPH + methanol) and A_{bs} is the Absorbance in presence of sample (DPPH + sample)

Antioxidant activities of leaf and callus extracts of *A. serratus* have been also expressed in term of IC_{50} values. An IC_{50} value is the specific concentration of extract that caused 50% degradation of DPPH radicals (30).

2.5.2. Ferric reducing antioxidant power assay (FRAP)

The FRAP assay was carried out according to the previously described method (31) with some modifications. Stock solutions 300mM acetate buffer (3.1 g $C_2H_3NaO_2 \times 3H_2O$ and 16ml $C_2H_4O_2$), pH 3.6, 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3 \times 6H_2O$ solution were prepared. Blank solution was prepared using distilled water. Both blank (distilled water) and 100 $\mu g/mL$ of leaf and callus extracted samples (0.20 ml) were allowed to react with 3.00 ml of the

FRAP working solution and incubated for 30 min in the dark at 37°C. The absorbance readings of the colored solution (ferrous tripyridyltriazine complex) were then taken against blank at 593 nm. Aqueous solutions of $FeSO_4 \cdot 7H_2O$ at concentrations 200, 400, 800, 1200 and 1600 μM were used as a standard solution and used to draw a standard curve ($R^2 = 0.991$). Results obtained were expressed as mg ferrous equivalent Fe (II) / gram of dry mass of extract. All determinations were performed in triplicate ($n = 3$).

2.6. Statistical Analysis

All the experiments were carried out in triplicate. The data are expressed as the mean \pm SD. The differences were analyzed by one-way Analysis of Variance (ANOVA) using SPSS statistical software (SPSS 20.0.) and Dunnett's *t*-test was used as the test of significance of the difference between the means. P value < 0.05 was considered as the minimum level of significance.

3. Results and Discussions

3.1. Callus Induction

Development of callus from leaf explants of *A. serratus* was observed after 10 to 12 days of culture. The calli produced were creamy white in color and friable in nature (Fig. 1). The calli when sub cultured on the same media proliferated and increase in mass. These calli were collected and air dried in the dark and used for phytochemical study.



Figure 1. Callus samples for phytochemical study. A, B, C and D: calli induced and grown on MS media. E; callus collected and F; Calli air dried for phytochemical study.

3.1.2. Extraction Yield

The leaf and leaf derived callus of *A. serratus* were extracted using solvents such as Water, Methanol, Ethyl acetate, Chloroform, and Petroleum ether by Soxhlet extraction (Fig.2). The percentage yield (weight) of each solvent extracts are presented in Table- 1. Methanolic

extracts of both leaf and callus exhibited higher yield 15.4% and 8.9% respectively and followed by water extracts 11.3% and 7.6% respectively. Petroleum ether leaf and callus extract showed the lowest yield of 6.20% and 2.4% respectively. Generally callus extracts showed lower yield than that of leaf extracts in all the solvents.

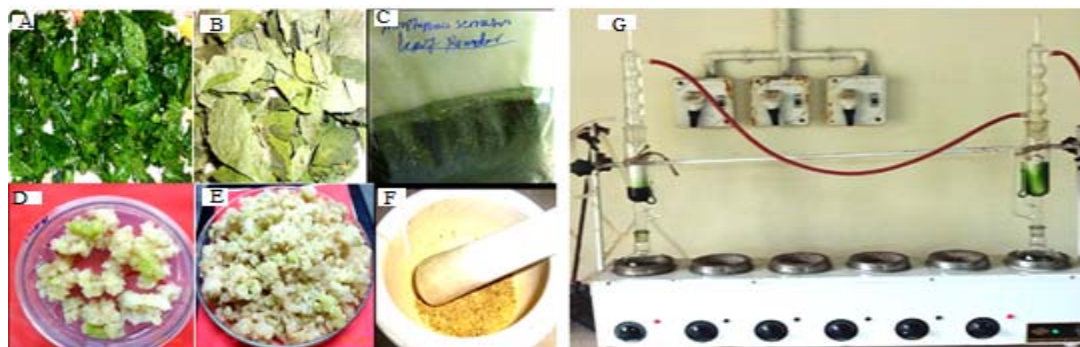


Figure 2. Leaf and callus samples soxhlet extraction: A, B and C leaf samples washed, dried and powder, D,E and F Callus tissue dried and powdered, G) Soxhlet extraction.

Table 1: The percentage yield (weight) of each solvent extracts of leaf and leaf callus of *A. serratus*.

Types of Extract	Yield (% w/w)		Appearance	
	Leaf	Callus	Leaf	Callus
Water	11.30	7.60	Yellow, non sticky	Yellow, non sticky
Methanol	15.40	8.90	Brownish black, sticky	Yellow, non sticky
Ethyl acetate	9.80	4.00	Brownish green sticky	Yellow, non sticky
Chloroform	6.90	3.6	Brown sticky	Yellow, non sticky
Petroleum ether	6.20	2.4	Oily, greenish black	Yellow, non sticky

3.2. Preliminary phytochemical screening

The extracts obtained from the different solvents were subject to various preliminary phytochemical tests for the identification of different constituents in leaf and leaf derived callus of *A. serratus*. The qualitative phytochemical analysis showed the presence of phytochemical constituents such as alkaloids, terpenoids, phenols, steroids, glycosides (flavonoids, saponins, tannins,), and quinones (Table.2). The result showed that all the tested phytochemicals were found in both leaf and leaf callus extracts but different solvent extracts of leaf and callus showed varied results. The aqueous and methanol extracts indicates the presence of all tested

phytochemicals. Ethyl acetate extract revealed the presence of all but not steroids and tannins. The extracts of chloroform gave positive results for only flavonoids, phenols and steroids. That of petroleum ether gave positive result for alkaloids, phenols, steroids saponins and tannins.

The ethyl acetate callus extract contains alkaloids, terpenoids, phenols, steroids, saponins, glycosides, proteins and carbohydrates. The petroleum ether extract indicates the presence of flavonoids, phenols, steroids, proteins and carbohydrates while the chloroform extract of callus indicate the presence of phenols and flavonoids only (Table 2).

Table 2: Qualitative Phytochemical Screening of Various solvent extracts of leaf and callus of *A. serratus*

Phytochemical		Leaf Extracts					Callus extracts				
		WA	ME	EA	CH	PE	WA	ME	EA	CH	PE
Alkaloids	Mayer's test	+	+	+	-	+	+	+	+	-	-
	Wagner's test	+	+	+	-	+	+	+	+	-	-
Flavonoids	Lead acetate test	+	+	+	+	-	+	+	+	+	+
	Ammonium solution test	+	+	+	+	-	+	+	+	+	+
Terpenoids	Salkowski's test	+	+	+	-	-	+	+	+	-	-
Phenolics	Ferric chloride test	+	+	+	+	+	+	+	+	+	+
	Lead acetate test	+	+	+	+	+	+	+	+	+	+
Steroids	Salkowski's test	+	+	-	+	+	+	+	+	-	+
	Lieberman and Burchard's test:	+	+	-	+	+	+	+	+	-	+
Saponins	Foam test	+	+	+	-	+	+	+	+	-	-
Tannins	Ferric Chloride Test	+	+	-	-	+	+	+	-	-	+
	Lead acetate test	+	+	-	-	+	+	+	-	-	+
Glycosides	Liebermann's test	+	+	+	-	-	+	+	+	-	-
	Salkowski's test	+	+	+	-	-	+	+	+	-	-
	Keller-Kiliani's test	+	+	+	-	-	+	+	+	-	-

WT= Water extract, **ME** = Methanol extract, **EA**= Ethyl acetate extract, **CH** = Chloroform extract, **PE**= Petroleum ether extract, + indicates presence of the phytochemical and – indicates absence of the phytochemical

The results of this study were partially in agreement with the observation obtained by other researches; (32) reported the presence of phenolics, flavonoids, tannis, saponins in the crude extract of leaf of *A. serratus* by UV spectrophotometer analysis. No previous study was available on the phytochemical constituent of the callus from this plant. But there are reports on the same phytochemicals constituent of the callus and the mother plant in other plants. For example, (33) reported that the preliminary phytochemical screening of leaf and callus *Solanum nigrum* revealed that the leaves contain alkaloids, glycosides, phenol, saponins, tannins and flavonoids whereas, glycoside saponins, phenol and flavonoids were found in callus of the plant. Other report also revealed that the acetone extract of leaf *A. serratus* showed a high amount of phenolic content 0.73 and 0.82% in both cold and hot extracts respectively (31). This is much lower than the result obtained in this study. This might be due to the solvent used for the extraction or the extraction method used. The result of this study also

confirmed not only this, but the methanolic extract contains a high amount of phenols. Also results from this study showed that the callus also contains phenols in fewer amounts than the leaf.

The qualitative phytochemical screening studies of leaf and callus extracts of *A. serratus* especially water and methanol extracts revealed the presence of almost all important bioactive compounds such as alkaloids, phenols, steroids, flavonoids, saponins, tannins, glycosides. These phytochemicals compounds present in the leaf and callus extracts are responsible for many biological activities (34). The presence of these phytochemicals strengthens the traditional use of the plant *A. Serratus* have medicinal value. The presence of alkaloids, saponins and tannins in plants play very important role in pharmaceutical field as reported (35). These compounds have various antibiotic properties and they are used in treating diseases caused by common pathogenic strains.

3.3. Quantitative analysis of phytochemicals

The results of quantitative estimation of five major groups

of phyto-constituents such as alkaloids, phenols, flavonoids, saponins and tannins in the leaves and callus extracts showed

that total phytochemical constituent of leaf extracts were higher compared to that of callus (Table 3).

Table 3: Quantitative analysis of phytochemical constituents of *A. serratus* leaves and callus extracts.

Extract	Total Alkaloids Content (mg/gm of extract)		Total phenols Content (mg GAE/gm of extract)		Total Flavonoids Content (mg GAE/gm)		Total Tannin content(mg GAE/gm of extract)		Total Saponins Content (mg/gm)	
	Leaf	Callus	Leaf	Callus	Leaf	Callus	Leaf	Callus	Leaf	Callus
W	14.37±0.31	3.80±0.30	78.86±4.12	40.05±8.62	57.17±6.35	29.47±4.15	56.49±9.85	39.08±1.56	69.34±0.19	29.55±2.22
M	16.37±0.76	6.90±0.80	104.73±4.3	54.42±6.59	66.86±4.15	39.17±6.35	69.59±1.27	49.37±5.40	32.91±1.54	22.25±4.42
E	12.77±0.40	1.37±0.21	73.11±8.98	27.11±4.31	69.63±6.35	26.09±4.15	---	---	22.96±1.40	13.49±1.49
C	---	---	42.93±4.98	22.80±4.31	58.55±4.15	14.24±2.40	28.28±6.42	---	16.94±0.25	---
P	12.77±0.40	1.37±0.21	42.93±4.31	22.80±6.59	---	8.71±4.15	28.28±2.49	21.23±0.90	18.03±0.26	9.93±1.12

Results are presented as the mean of three analyses ± standard deviation (n=3 ±SD); W: water extract, M: methanol extract, C: chloroform extract, E: ethyl acetate extract, P: petroleum ether extract, GAE: Gallic Acid Equivalent.

3.3.1. Determination of alkaloids

The total alkaloids content analysis in leaf and callus extracts of *A. serratus* exhibited that alkaloids content is higher in leaf extract than in callus. The total alkaloids content ranges from 12.77±0.40 to 16.37±0.76 and 1.37±0.21 to 6.90±0.80 mg /g in the leaf and callus extracts respectively (Table 3 and Figure 5.3). In both leaf and callus, methanol extracts exhibited the highest alkaloids content 16.37±0.76

and 6.90±0.80 mg/ g respectively followed by water extract which exhibited 14.37±0.31 and 3.80±0.30 mg/g respectively. The chloroform extracts of both leaf and callus did not show the presence of alkaloids. In both leaf and callus, the ethyl acetate extracts and the petroleum ether extracts showed the same alkaloids contents 12.77±0.40 mg/g and 1.37±0.21mg/g respectively.

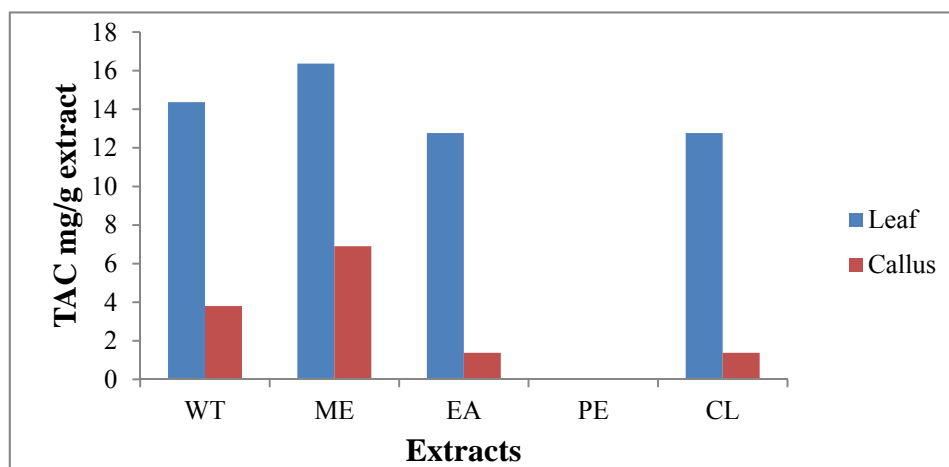


Figure 3: Total Alkaloids Content in the leaf and callus extracts in different solvents. TAC= Total Alkaloids content, WT=Water, ME=Methanol, EA= Ethyl acetate, CL= Chloroform, PE=Petroleum ether.

3.3.2. Determination of total phenol content

The quantified phenolic contents were ranging from 42.93±4.98 to 104.73±4.3 and 22.80±6.59 to 54.42±6.59 mg GAE/g extract in leaves and callus respectively (Table 3 and Fig. 4). The methanol extract of both leaf and callus extracts gave the highest phenolic content 104.73±4.3 and 54.42±6.59 mg GAE/g extract respectively than that of other solvent extracts. The next higher phenolic content

78.86±4.12 mg GAE/g and 40.05±8.62 mg GAE/g was recorded in the water extract followed by 73.11±8.98 mg GAE/g and 27.11±4.31 mg GAE/g in the ethyl acetate extracts of both leaf and callus extracts respectively. The lowest phenol content was recorded in chloroform and petroleum extracts of leaves and callus than other solvents (Table 3 and Fig. 4).

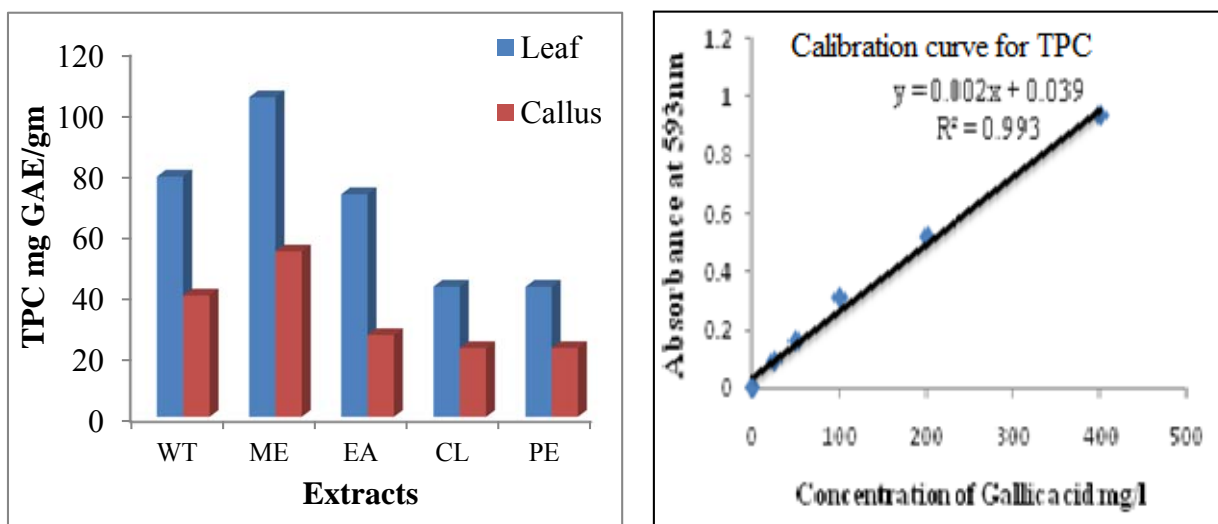


Figure 4: Total Phenolics Content of the leaf and callus extracts in different solvents. TPC= Total phenolics content GAE=Gallic acid equivalent, WT=Water, ME=Methanol, EA= Ethyl acetate, CL= Chloroform, PE=Petroleum ether

3.3.3. Determination of total flavonoid content

The total flavonoids content of various extracts varied widely. Ethyl acetate extract of leaf and Methanolic extracts of callus demonstrated higher total flavonoid contents (69.63±6.35 and 39.17±6.35 mg GAE/gm) respectively than other solvents (Table 3 and Fig. 5). In leaf, the second highest flavonoid content (66.86±4.15 GAE/gm) was observed in the methanol extract followed by 58.55±4.15 GAE/gm in chloroform extract. In callus, the second highest flavonoid content (29.47±4.15GAE/gm) was observed in the methanol

extract followed by 26.09±4.15 GAE/gm in ethyl acetate extract. Totally, the leaf has higher flavonoid content than the callus in all solvent extracts except the petroleum ether which did not show any flavonoid content in leaf whereas the least flavonoid content (8.71±4.15 GAE/gm) in callus (Table 3 and Fig.5).

Among the five quantified phytochemical components, flavonoids content was highest in the leaf methanol extract followed by alkaloids and phenolic compounds (Table 3 and Figure 3). The total content of tannins and saponins were low in the chloroform extract.

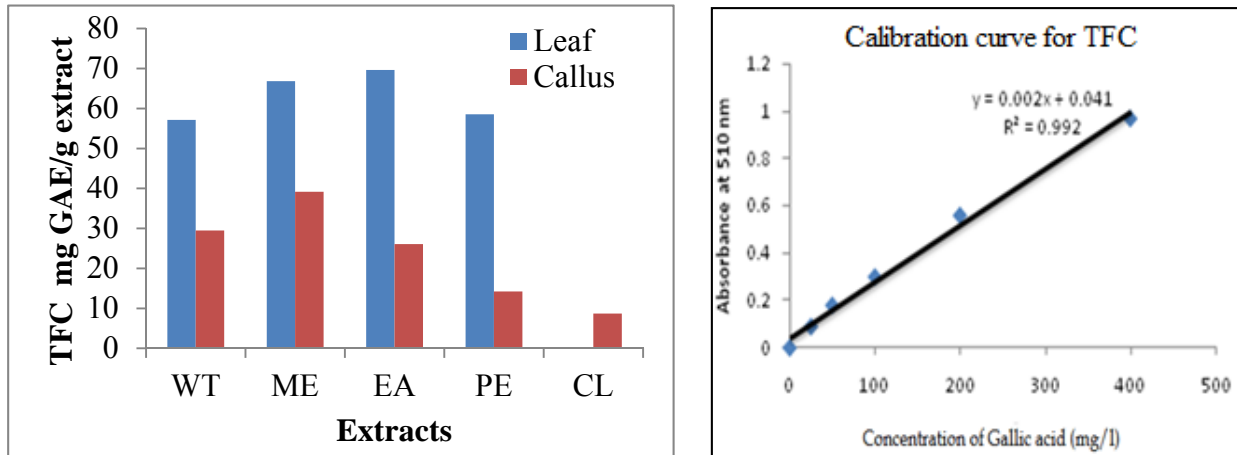


Figure 5: Total Flavonoids Content of the leaf and callus extracts in different solvents. TFC= Total flavonoids content GAE=Gallic acid equivalent, WT=Water, ME=Methanol, EA= Ethyl acetate, CL= Chloroform, PE=Petroleum ether

3.3.4. Determination of total tannins content

Total tannins content in leaves and callus extracts of different solvents range from 28.28±6.42 to 69.59±1.27 mg GAE/g and from 21.23±0.90 to 49.37±5.40 mg GAE /g respectively. The methanol extract of both leaf and callus depicted high tannins content (69.59±1.27 mg GAE/g and 49.37±5.40 mg GAE/g respectively) than other extracts. The ethyl acetate extract of leaf and the ethyl

acetate and chloroform extracts of callus do not contain tannins (Table 3 and Fig.6). The highest tannin contents 69.59±1.27 mg GAE/g in leaf 49.37±5.40 mg GAE /g in callus were recorded in methanol extracts in both. The petroleum extract of both leaf and callus showed the lowest tannin contents 28.28±6.42 mg GAE/g and 21.23±0.90 mg GAE/g respectively. In general, the leaf extracts showed high tannin contents than the callus extracts. (Table 3).

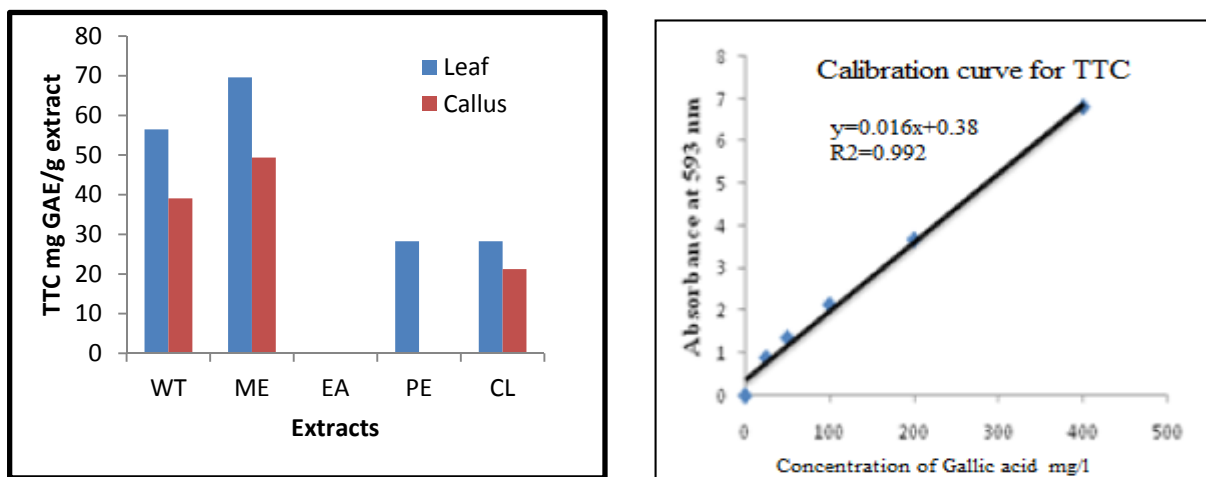


Figure 6: Total tannin content in the extracts of leaf and callus. TTC= Total Tannin content GAE=Gallic acid equivalent, WT=Water, ME=Methanol, EA= Ethyl acetate, CL= Chloroform, PE=Petroleum ether

3.3.5. Determination of total saponin content

The total saponins contents of leaf and callus extracts were ranging between 16.94±0.25 to 69.34±0.19 mg/ g and 9.93±1.12 to 29.55±2.22 mg/ g respectively (Table 3 and Fig 7). The water extract of leaf and callus exhibited a high content of saponins 69.34±0.19 mg/ g and 29.55±2.22 mg/ g respectively followed by methanol extracts which

showed 32.91±1.54 mg/ g and 22.25±4.42 mg/ g saponin contents respectively (Table 5 and Fig 5). The chloroform extracts of callus showed no saponin content, but that of leaf contains 16.94±0.25 mg/g saponin which was the least. In both callus, the petroleum extract exhibited the lowest saponin contents 9.93±1.12 mg/g (Table 3 and Fig 7).

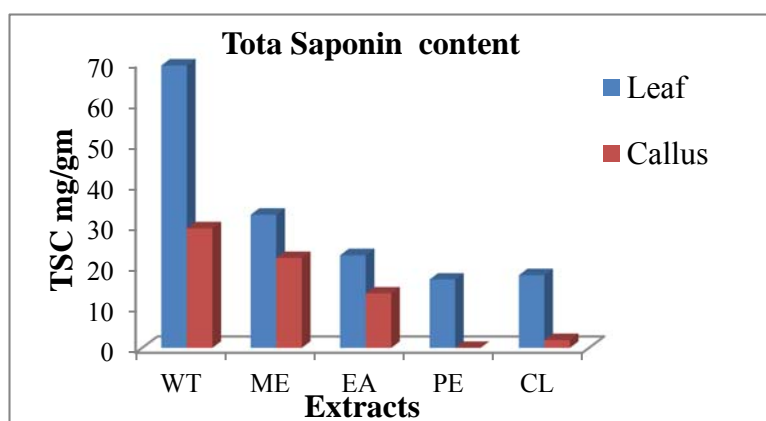


Figure 7: Total saponin contents in the extracts of leaf and callus. TSC= Total saponin content WT=Water, ME=Methanol, EA= Ethyl acetate, CL= Chloroform, PE=Petroleum ether.

Generally the results of quantitative analysis of phytochemicals suggested that higher yields of phenolics followed by flavonoids, soluble tannins saponins and alkaloids, (Table 3). There is no previous report on the total alkaloids content in the *A.serratus* leaf and callus extracts. The result of the present study showed the methanolic extracts contain higher alkaloids content in both leaf and callus. This may be due to environmental factors such as the type of media used to grow the callus. Environmental factors can influence the total quantities of secondary metabolites in callus (36). For example, (37) reported that callus developed on MS media fortified with 2, 4-D and BAP were most promising in relation to alkaloids content.

The phenolic contents of both leaf and callus polar solvent extracts were higher than those relatively non polar solvents. This may be due to high solubility of phenolics compounds more in polar solvents than non polar solvents (38). The difference in total phenolic content for each solvent was because of the polarities and solvating

strengths of different solvent (39). According to (40), the polarity of solvent is very important in increasing the solubility of phenolic compounds.

The result of this study is in agreement with (31) who reported the presence of high amount of flavonoids content in the acetone extract of *A. serratus*, but in contrast to (41) who reported higher values of flavonoids content in *Jatropha curcus* callus compare to *in vivo* plant. Other report also revealed the presence of flavonoids in leaf derived callus of *Saussurea medusa* Maxim (42). The methanolic extract showed high flavonoid content which is in line with the study which showed that flavonoids present in the plant *A. serratus* might be present in the glycosidic form as the more polar solvent shown, the higher content than the less polar solvent extract (31).

3.4. Antioxidant activity Assessment

3.4.1. DPPH radical scavenging activity

The results of DPPH radical scavenging activity of leaf

and callus extracts with known antioxidant standards Ascorbic acid are shown in Table 4. 100-800 µg/ml of the water, methanol, ethyl acetate, chloroform and petroleum extracts produced moderate to high DPPH scavenging activity in both extracts. Overall results indicate that all leaf extracts showed higher percentage scavenging activity than the callus. At all concentrations, the methanolic extract showed comparable antioxidant activity with the standard ascorbic acid compared to other extracts. The methanolic leaf extract exhibited highest antioxidant activity (74.70±0.12 and 61.85±0.03 at concentrations of 600 and 800 µg/ml respectively) followed by water leaf extract (53.41±0.41 at concentration of 800 µg/ml)(Table 4). From callus extracts the methanol extract showed the highest percent inhibition (48.59±0.03 at a concentration of 800 µg/ml followed by same extract 42.17±0.02 at a concentration of 600 µg/ml). The least percent inhibition (3.61±0.05) was recorded by water callus extract at a concentration of 100µg/ml (Table4). The DPPH scavenging activity of both leaf and callus increases with

decreasing the polarity of the solvent. The DPPH radical scavenging activities of the extracts were also concentration dependent which increases as the concentration of the extracts increased. Based on the study results the more polar water and methanol extracts have more effective antioxidants compared to others.

The concentration of the leaf and callus extracts that degrade 50% of DPPH in the solution (IC₅₀) was also calculated (Table 4 and Fig. 8). IC₅₀ value is inversely related to the activity as it is the measure of inhibitory concentration and a lower value would reflect greater antioxidant activity of the fraction. Methanol leaf extracts exhibited lowest IC₅₀ value (429.38±0.03 µg/ml) compared to other extracts. Water and ethyl acetate leaf extracts showed IC₅₀ value (662.62±0.31 and 807.14±0.04µg/ml) respectively. The IC₅₀ values of methanol and water callus extracts show (750.40±0.04, 811.80±0.05µg/ml) respectively relative to an Ascorbic acid having IC₅₀ of 152.22±0.32 µg/ml (Table 4 and Fig. 8).

Table 4: DPPH inhibition (%) and IC₅₀ in leaf and callus extracts on Ascorbic acid as standard

Standard and sample		% inhibition					IC ₅₀
		100	200	400	600	800	
Ascorbic acid		60.24±0.01	73.49±0.00	86.75±0.02	87.95±0.01	89.96±0.10	152.22±0.32
Water extract	Leaf	14.86±0.31	24.10±0.20	36.55±0.03	51.00±0.03	53.14±0.12	662.62±0.31
	Callus	3.61±0.05	10.04±0.03	17.27±0.04	28.51±0.03	37.35±0.00	1071.62±0.05
Methanol extract	Leaf	23.69±0.05	50.20±0.01	52.61±0.02	61.85±0.03	74.70±0.12	429.38±0.03
	Callus	25.30±0.01	26.91±0.01	37.75±0.03	42.17±0.02	48.59±0.03	750.40±0.04
Ethyl acetate	Leaf	18.47±0.04	28.11±0.01	34.54±0.30	39.36±0.03	46.18±0.03	807.14±0.04
	Callus	8.43±0.04	32.53±0.02	30.92±0.03	31.33±0.04	36.55±0.02	811.80±0.05
Chloroform extract	Leaf	18.07±0.03	23.29±1.00	26.51±2.40	40.96±0.03	42.57±0.03	882.98±1.05
	Callus	7.23±0.03	15.66±0.03	18.07±0.01	24.90±0.03	30.92±0.01	1323.14±0.21
Petroleum ether extract	Leaf	15.26±0.04	19.68±0.03	27.31±0.03	30.92±0.00	35.74±0.01	1107.63±2.02
	Callus	9.64±0.04	12.05±0.03	12.05±0.01	17.67±0.02	25.30±0.03	1852.80±

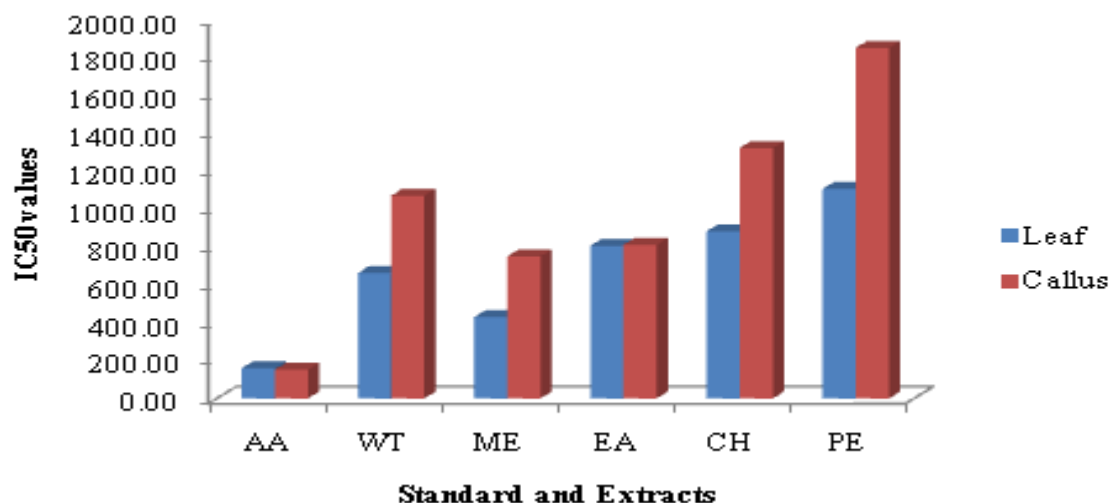


Figure 8: IC₅₀ value of DPPH antioxidant activity of leaf and callus extracts. AA=Ascorbic acid, WT= Water extract, ME= Methanol extract, EA= Ethyl acetate extract, CH= Chloroform extract, PE= Petroleum ether extract.

It is known that the presence of phenolic compounds hydroxylgroups of plants is responsible for their DPPH radical scavenging activity. The antioxidant activity of plants is mainly due to the presence of phenolic which has redox properties and allow them to act as hydrogen donors, reducing agents, and singlet oxygen quenchers.

The increase in scavenging activity with decreasing the polarity is in agreement with the report of (43) which revealed that the ethanol and methanol extracts which are more polar solvent extracts, were more efficient antioxidants compared to the non-polar hexane extract in DPPH assay. The present results are also in agreement with the report of (44), that antioxidant activity of methanolic extract of the *in vivo* grown *Spilanthes acemella* is higher than the *in vitro* grown callus.

The observed antioxidant activities of the leaf and callus extracts of *A. serratus* could be due to the presence of polyphenolic compounds (45). From the observed results it can be suggested that *A. serratus* leaf and callus extracts might serve as a effective therapeutic agent for free radical scavenging and the regulation of pathological conditions due to oxidative stress.

3.4.2. FRAP antioxidant assay

From the leaf extracts, the highest and the lowest FRAP values (663.67 $\mu\text{M Fe}^{+2}/\text{g dw}$ and 220.33 $\mu\text{M Fe}^{+2}/\text{g dw}$) were recorded in methanol extract and petroleum ether extract respectively (Table 5). From the callus extracts, the highest and the lowest FRAP values (445.67 $\mu\text{M Fe}^{+2}/\text{g dw}$ and 27.00 $\mu\text{M Fe}^{+2}/\text{g dw}$) were recorded in methanol extract and petroleum extract respectively. Methanol was found to be the best extraction solvent for FRAP study of *A. serratus* leaf and callus which showed the maximum reducing power. Water extracts showed the second highest reducing power in both leaf and callus extracts followed by ethyl acetate, chloroform, and petroleum extracts. This result is in line with the pervious study which reported methanol: water (1:1) to be the best solvent for ABTS and FRAP study of different betel leaf variety (44). This study also revealed that the leaf extracts show higher FRAP values than the callus extracts. Higher FRAP values give higher antioxidant capacity because FRAP value is based on reducing ferric ion, where antioxidants are the reducing agent.

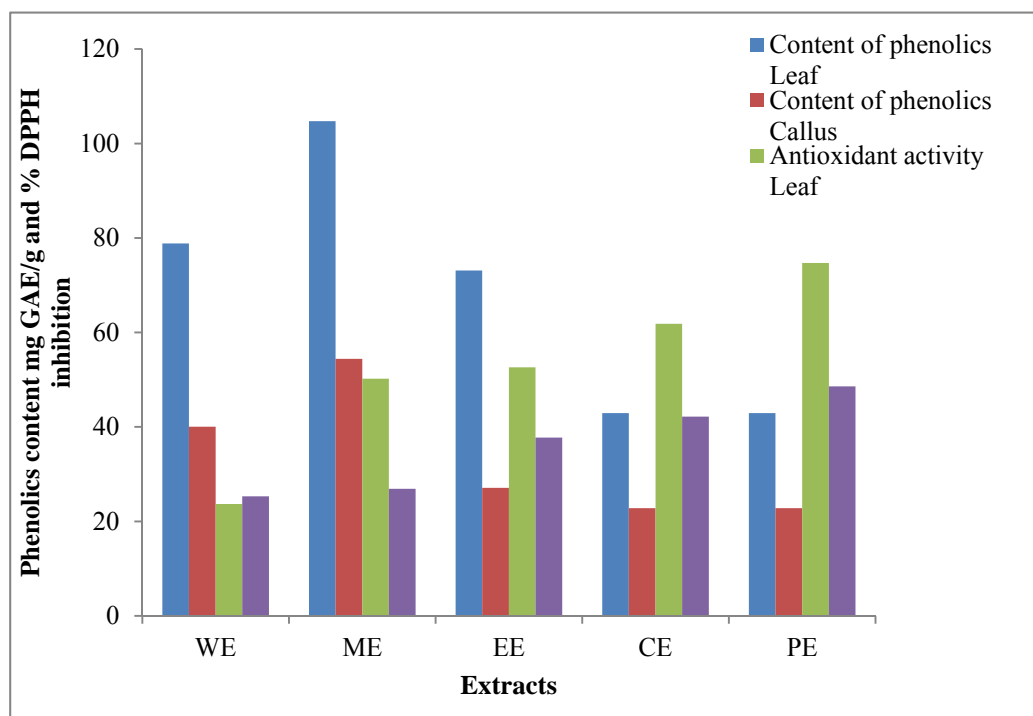
Table 5 :FRAP value of leaf and callus extracts on FeSO₄.7H₂O as standard

Sample	FRAP (Fe+2 μ M Eq./g dw basis)				
	Water	Methanol	Ethyl acetate	Chloroform	Petroleum ether
Leaf	597.00 \pm 2.25	663.67 \pm 0.97	303.67 \pm 1.21	230.33 \pm 0.53	220.33 \pm 0.22
Callus	330.33 \pm 0.91	445.67 \pm 0.32	160.33 \pm 1.22	67.00 \pm 0.32	27.00 \pm 0.47

3.4.3. Correlation between phenolic content and antioxidant activity

There is a correlation between antioxidant activities of leaf and callus extracts with the total phenolics and flavonoids content. The higher the total phenolics and flavonoid contents the higher the antioxidant activity of the extracts (Fig 9 and 10). The results are in agreement with the previous studies which reported that the free radical scavenging activity of

plant extracts correlate with the phenolic content (45). High positive relationship between total phenolics and antioxidant activity was also reported in many plant species (47). The result of this study also revealed that radical scavenging activity of leaf and callus extracts increased as the concentration of extract increased which is in agreement with (48) who reported that in *P. marsupium*.

**Figure 9: Correlation between DPPH radical scavenging activity and total phenolics content**

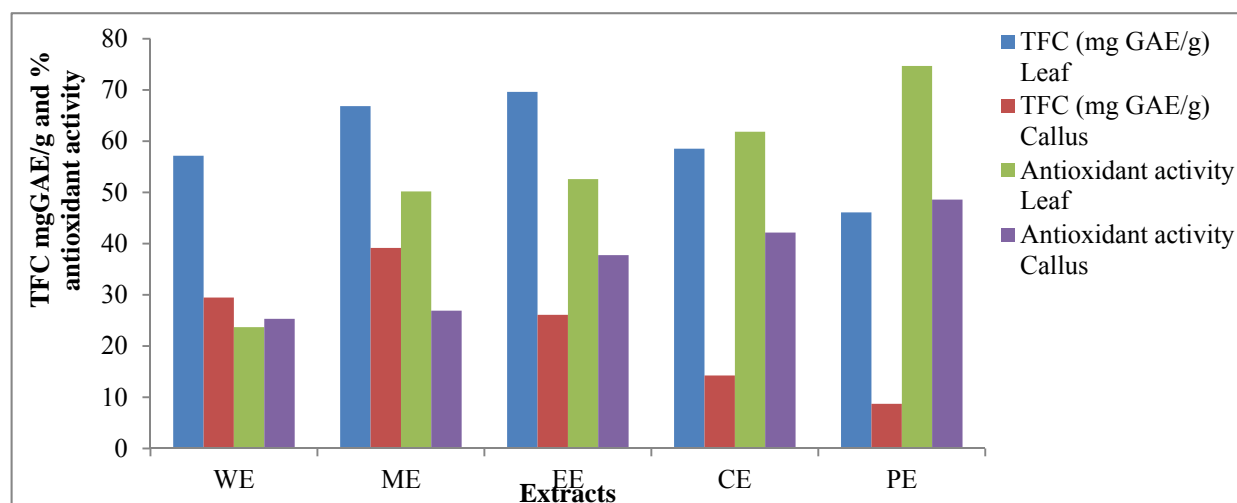


Figure 10: Correlation between DPPH radical scavenging activity and total flavonoid content. TFC= Total Flavonoids content GAE=Gallic acid equivalent, WT=Water, ME=Methanol, EA= Ethyl acetate, CL= Chloroform, PE=Petroleum ether

There is also a positive correlation between the reducing capability and antioxidant activity of the extracts. The DPPH inhibition percentage value and FRAP value show the same trend: the greater the extract reducing power, the greater the antioxidant activity. Similar trend was also reported in other plants such as pineapple, banana, and guava plants (49).

4. Conclusion

Plants provide an important source of world's pharmaceuticals that have proved to be the most useful in the treatment of diseases. The most important bioactive constituents of plants that are used as pharmaceuticals are phenols, steroids, terpenoids, carotenoids, alkaloids, glycosides (anthraquinones, flavanoids and tannins). Plants are very important valuable starting material for drug discovery and development. Essential information provided by qualitative and quantitative phytochemical screening of plant extracts regarding the chemical constituents help for the pharmacological as well as the pathological discovery of novel drugs.

The qualitative analysis results of the present study showed that both the leaf and callus extract of *A. serratus*

contains all phytochemical tested (alkaloids, phenolics, glycosides (flavonoids, saponins, tannins), steroids, and polyphenols. In quantitative analysis, all phytochemicals tested (alkaloids, phenols, flavonoids, tannins, saponins) showed that the callus tissue has the same phytochemicals as the leaf but in lower amounts. The leaf extracts contain highest amounts of phytochemical constituents compared to callus extracts. Therefore, other than the leaves itself, callus tissues could be an alternative source of secondary metabolites in *A. serratus*. However, further study is needed to increase production and to optimize the process. Similarly, leaf extracts, exhibited the highest antioxidant activity in comparison to callus extracts. Nevertheless, this work gives basic information for *in vitro* mass production of secondary metabolites as a natural source of antioxidants in *A. serratus*. The callus culture of medicinal plants can be used as a potent source of desired bioactive metabolites without destroying the rare/endangered wild plant resources. Since the phytochemical study of *A. serratus* intact plant and *in vitro* callus tissues are still limited, this study could be the base for further study and research on the production of secondary metabolites by *in vitro* culture.

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فحص المواد الكيميائية النباتية وتحليل نشاط مضادات الأكسدة في المختبر لمستخلصات الأوراق والكالس من *Allophylus serratu*

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ملخص

Allophylus serratus هو نبات طبي مهم تستخدم أوراقه وثماره وجذوره وساقه في الأدوية التقليدية. تهدف الدراسة الحالية إلى إجراء تحليل كيميائي نباتي أولي وتحديد النشاط المضاد للأكسدة لمياه الأوراق والكالس والميثانول وخلات الإيثيل والكلوروفورم ومستخلصات الإيثر البترولي من *A. serratus*. خضعت المقطعات لاختبارات نوعية وكمية مختلفة لتحليل الكيمياء النباتية. تم فحص نشاط مضادات الأكسدة في المختبر من المستخلصات بواسطة (2-DPPH، 2-diphenyl-1-picrylhydrazyl، ABTS ++، 2)، (FRAPS (تقليل الحديدك / القوة المضادة للأكسدة) المقاييس. أكد التحليل الكيميائي النباتي النوعي وجود قلويدات، الفينولات، الفلافونويد، الجليكوسيدات، الصابونين، التانينات، والستيرويدات في مستخلصات الأوراق والكالس. أظهرت نتائج التحليل الكمي إنتاجية أعلى من الفينولات (4.3 ± 104.73 مجم) تليها الفلافونويد (6.35 ± 69.63 مجم) والعص القابل للذوبان (1.27 ± 69.59 مجم) مكافئ حمض الجالليك / جرام من الميثانول وخلات الإيثيل والميثانول على التوالي. في حالة مستخلصات الكالس، فإن أعلى إنتاج هو الفينولات (6.59 ± 54.42 جم) تليها الفلافونويد (39.17 ± 6.35 جم) مكافئ حمض الجالليك / جم من مستخلصات الميثانول. أظهرت فحوصات النشاط المضاد للأكسدة لكل من مستخلصات الأوراق والكالس نشاطاً قوياً مضاداً للأكسدة والذي يمكن أن يكون بسبب المحتوى العالي من الفينولات والفلافونويدات. أظهر مستخلص الميثانول إمكانات مضادة للأكسدة والذي يمكن أن يكون بسبب المحتوى العالي من الفينولات والكالس، لكن مستخلصات الأوراق تظهر نشاطاً كبيراً كمضاد للأكسدة مقارنة بمستخلصات الكالس. لذلك، تحتوي كل من مستخلصات الأوراق والكالس من *Allophylus serratus* على مواد كيميائية نباتية تعد مصدراً محتملاً لمضادات الأكسدة مما يشير إلى أن النبات يمكن أن يكون بمثابة مصدر للأدوية المفيدة.

الكلمات الدالة: *Allophylus serratus*، نشاط مضاد للأكسدة، فحص كيميائي نباتي، مستخلص الكالس.

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