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PHYTOCHEMICAL SCREENING AND *IN-VITRO* ANTIOXIDANT ACTIVITY ANALYSIS IN LEAF EXTRACT OF WATER APPLE [*SYZYGIUM AQUEUM* (BURM. F) ALSTON]

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Keywords:

Syzygium aqueum, Total phenol, Flavonoid and antioxidant activity, Water apple

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ABSTRACT: *Syzygium aqueum* is a well-known edible fruit-bearing plant species. The tribal people in Malaysia used different parts of the plant to cure various ailments. The previous studies on phytochemical evaluation of *S. aqueum* are very limited. The present study aims to conduct a detailed investigation of phytochemicals present in the leaves of *S. aqueum* and also its potential use as an effective antioxidant agent. In this study, the methanolic leaf extract of *S. aqueum* was tested to determine its chemical composition and its antioxidant activity. The result of the preliminary phytochemical investigation in leaf extract of *S. aqueum* revealed the presence of several bioactive secondary metabolites such as phenolic compounds, flavonoids, tannins, steroids, and terpenoids. Quantification results showed that the total phenol was found to be 62.89±1.48% GAE and the flavonoid content was 15.05±0.36% QE. Further analysis of the antioxidant activity in leaf extract of S. aqueum using different antioxidant assays proved that the extract effectively scavenging diverse types of free radicals. In different antioxidant assays, the leaf extract showed different IC_{50} values *viz.*, an IC_{50} of 83.53 µg/mL in 1, 1-diphenyl-2-picryl- hydrazyl (DPPH) scavenging assay, an IC_{50} of 721.59 µg/mL in Hydrogen peroxide radical scavenging assay, an IC_{50} of 1338.73 µg/mL in Superoxide radical scavenging assay, an IC_{50} of 3268.99 µg/mL in Nitric oxide radical scavenging activity, an IC_{50} of 1441.0 µg/mL in Hydroxyl radical scavenging assay, an IC_{50} of 2802.521 µg/mL in ABTS scavenging assay.

INTRODUCTION: In an *in-vivo* system, oxidation is very much essential to produce metabolic energy. However, as a result of an imbalance in metabolic reactions, there could be a generation of oxygen-centered free radicals and other reactive oxygen species that can cause cell death and tissue damage.

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Oxidative stress is considered a major causative factor in the induction of many chronic and degenerative diseases. The frequency of oxidative damage is more in older age and also in a disease condition. All higher living organisms, including humans, are well protected against reactive oxygen species by naturally occurring antioxidant compounds or by several inborn mechanisms associated with them [1].

An antioxidant is a chemical compound that prevents or delays the oxidation of other chemical substances [2]. Generally, antioxidant compounds are classified as enzymatic and non-enzymatic anti-oxidants.

Most well-known antioxidant compounds like phenolic acids, polyphenols, and flavonoids scavenge free radicals such as peroxide, hydrogen peroxide and thereby inhibit the oxidative mechanism that leads to degenerative diseases. A recent report on antioxidant protection of living organisms suggested that intake of antioxidant rich diet is inversely associated with the risk of some lifestyle diseases [3-4]. Hence the recent trend is to search for naturally occurring antioxidant-rich foods with a special interest in those natural foods that are commonly available. *Syzygium aqueum* is one such plant species belonging to the scope of antioxidant-rich natural food resources.

Another trend in plant phytochemical research lies on the identification of naturally occurring plant-derived antiseptic agents. The antiseptic properties of medicinal plants and their extract have been well established since antiquity, while they attempt to prove these properties dates back to early 1900 [5]. Although *Syzygium aqueum* is a well-known plant species belonging to the Family Myrtaceae, the antiseptic property of *S. aqueum* is not yet well known for our society. The species is widely distributed in the tropical and subtropical region of the world and is considered as native to Malaysia and Indonesia. The plant is formerly known as *Eugenia aquea* bearing edible fruits and is often used as a dietary supplement. The fruit of the plant is enriched with high water content; hence it got the name 'Water apple". Literature review regarding medicinal use of this plant revealed that in Malaysia, various parts of this plant had been used in traditional medicine. *S. aqueum* leaves possess antibiotic activity and the tribal people in Malaysia often ate raw leaves to get relief from birth pain [6]. The tribal people also used powdered dried leaves of this plant for the treatment of mouth ulcers, and preparation from its root has been used to relieve itching and swelling. A decoction made from the bark of *S. aqueum* act as an effective astringent [7]. A recent report regarding the cosmeceutical potential of leaf extract of this species proved its utility as a cosmetic ingredient due to its antityrosinase, lipolytic, and anticellulite activities [8]. Literature review of this species also revealed that its leaves possess anticancer and antidiabetic activity [9]. The medicinal properties of this plant are mainly due to the existence of its bioactive phytochemicals. Most of these phytochemicals can act as powerful antioxidant and antimicrobial agents. Therefore, the present study was designed to detect various phytochemicals present in leaf extract of *S. aqueum* and its use as a strong antioxidant agent.

MATERIALS AND METHODS:

Material Collection: The mature leaves of *S. aqueum* were collected from fully grown trees located in the Thiruvananthapuram district. The collected plant samples were deposited at the Herbarium of Botany department in the University of Kerala with voucher number KUBH 10278. The collected leaves were washed under running tap water, and the final round of cleaning was done using double distilled water. The cleaned leaves were dried at room temperature. After drying, the leaves were powdered using a mixer grinder. The powdered dried leaves were then subjected to Soxhlet extraction.

Equipment: The quantitative analysis of total phenols, flavonoids, and antioxidant tests were performed spectrophotometrically using SHIMADZU UV-1800, UV-Visible Spectrophotometer.

Chemicals: All chemicals used were in analytical grade and were purchased from Sigma Aldrich.

Extract Preparation: The shade-dried powdered leaves were subjected to Soxhlet extraction using methanol as a solvent system. Since methanol is highly polar in nature, the solvent can easily elute most of the phytochemicals present in leaves of *S. aqueum.* The collected extract was then dried in Vacuum rotary evaporator and the yield percentage was calculated using the following formula.

Yield percentage = (Weight of the extract / Weight of the Sample) × 100

The dried extract was stored in airtight glass bottle and kept in refrigerator for further analysis.

Preliminary Phytochemical Screening: The methanolic leaf extract was tested for the presence of major secondary metabolic compounds using standard procedure [10-11].

Quantification:

Determination of Total Phenolic Content (TPC): Total phenolic content (TPC) of methanolic extract was determined using standard protocol [12]. The extract was initially prepared in 1mg/10 mL concentration. From the stock, 0.1 mL of the

extract was pipetted out into a test tube. To this solution, 3.9 mL of the distilled water and 0.5 mL of Folin's reagent were added. Later, the mixture was incubated at room temperature for 3 min and finally 2 mL of 20% sodium carbonate solution added to this mixture. The solution was then kept in boiling water bath for 5 minutes and absorbance of the solution read at 760 nm. Gallic acid was used as a reference standard and the results were expressed as mg % of Gallic acid equivalent, GAE.

Determination of Total Flavonoid Content (TFC): Total flavonoid content of leaf extract was determined using a standard procedure [13]. The stock of the extract was initially prepared with 10mg/10mL concentration. From this initial stock, 1mL extract was pipetted out. To this solution, 75µL of 5% sodium nitrite was added and kept it for 6 min. After the 6 min, 150µL of 10% aluminum chloride was added to this mixture and kept it again undisturbed for 5 min. To this mixture, 0.5 mL of 1M NaOH was added. Then the final volume was made up to 2.5 mL using distilled water. The solution was then mixed thoroughly. Subsequently, the absorbance at 510 nm was measured with quercetin as a reference standard. The result was expressed in mg% of Quercetin equivalent (QE).

Antioxidant Assays:

DPPH Assay: The hydrogen atom donating or electron-donating ability of the extract were calculated from the bleaching of a purple-colored methanol solution of 2, 2- Diphenyl-1-picryl-hydrazyl (DPPH).DPPH activity of methanolic leaf extract was determined spectrophotometrically [14]. For doing this assay, the leaf extract was initially prepared at 1mg/mL concentration. Then, 0.1mM DPPH solution was prepared by dissolving 1.9mg DPPH in100 mL methanol. Different volume (12.5µL-200µL) of methanol extracts were added to 4% DPPH (0.1mM). The solutions were then kept in the dark. After 30 min of incubation in room temperature, the absorbance was read against the blank at 517 nm. The inhibition percentage was calculated using the following formula.

$$\% \text{ of Inhibition} = Abs_{control} - Abs_{sample} \times 100 / Abs_{control}$$

$Abs_{control}$ is the absorbance of control/blank solution (containing all reagent except the test sample). Abs_{sample} is the absorbance of the sample extract.

ABTS Assay: The ABTS radical scavenging activity of the extract was determined spectro-photometrically using a standard procedure [15]. The stock solution was prepared by mixing 0.3 mL of 17mM potassium persulfate solution in 50 mL of 20Mm ABTS solution. The reaction mixture was kept in the dark for 12 h before use. ABTS assay was performed by adding 1 mL of distilled water to various concentrations of samples. To this mixture, 0.16 mL of ABTS solution was added, thereby making the final volume of the mixture 1.36 mL. The absorbance was then measured Spectro-photometrically at 734nm after 20 min. Ascorbic acid was used as a reference standard.

Hydroxyl Radical Scavenging Activity: Hydroxyl radical scavenging activity was done by a standard procedure [16]. The assay is based on the condition or qualification of degradation product of 2-deoxyribose by condensation with TBA. In this assay, hydroxyl radical was generated by the Fe^{3+} - ascorbate- EDTA $–H_2O_2$ system (The Fenton reaction). The final volume of reaction mixture was 1 mL containing 2- deoxy- 2- ribose (2.8mM), $FeCl_3$ (100µm), EDTA (100µm), H_2O_2 (1.0mM), ascorbic acid (100µm) in 20mM KH_2PO_4 - KOH buffer having pH 7.4 and various concentrations (125-2000 µg/µl) of the test sample. After the incubation of the reaction mixture for 1 h at 37°C, 0.5 mL of the reaction mixture was pipetted out. To this solution, 1ml of 2.8% TCA and 1ml of aqueous TBA was added and incubated at 90 °C for 15 min to develop the color. After cooling, the absorbance was measured spectrometrically at 532nm. Gallic acid was used as a reference standard to determine the hydroxyl radical scavenging activity of the extract.

Hydrogen Peroxide Scavenging Activity: Hydrogen peroxide radical scavenging activity of the extract was determined using a standard method [17]. A 40mM solution of Hydrogen peroxide was prepared in phosphate buffer (pH 7.4). Then, 0.6 mL solution of $H_2O_2$was added to different concentrations of the extract. The mixture was incubated for 10 min. After the incubation, the absorbance of the solution was read at 230 nm. The percentage of Hydrogen peroxide scavenging activity was calculated using the following formula:

% of H_2O_2 scavenging effect = $A_{Control}$ - A_{Sample} × 100 / $A_{Control}$

Where $A_{Control}$ is the absorbance of Control and A_{Sample} is the absorbance of Sample.

Nitric oxide Scavenging Activity: Nitric oxide scavenging activity was measured spectrometrically using a standard procedure [18]. Different concentration of the extract was mixed with 5mmolL^{-1} solution of sodium nitroprusside prepared in phosphate buffer (pH 7.4) and incubated at 25°C for 30 min. A control was prepared by mixing methanol, and 5mmolL^{-1} solution of sodium nitroprusside prepared in phosphate buffer. After 30 minutes, 1.5 mL of incubated solution was taken and diluted with 1.5 mL Griess reagent (1% sulphanilamide, 2% phosphoric acid, and 0.1% N-1-naphthyl ethylene di-amine di-hydrochloride). The absorbance of the chromospheres formed during diazotization of the nitrate with sulphanilamide and subsequent coupling with N-1 naphathyl ethylene di-amine di-hydrochloride was measured at 546 nm. Gallic acid was used as a reference to the standard.

Superoxide Radical Scavenging Activity: Superoxide radical scavenging activity of the extract was determined by following a standard protocol described earlier [19]. Superoxide radicals were formed in the riboflavin-NADH system by the oxidation of NADH, and these radicals were assayed by the reduction of NBT, resulting in the formation of blue formazan. The test was conducted by mixing 0.02ml of sample, 0.05ml of 0.12mM Riboflavin, 0.2 ml of 0.1M EDTA and 0.1 ml of 1.5mM NBT (Nitro-blue tetrazolium) solution. The mixture was then diluted to 2.64ml with 0.067M phosphate buffer. The absorbance of the solution was measured after illumination for 30 min at 560 nm using a UV visible spectrophotometer. Ascorbic acid was used as a reference standard.

Total Reducing Power: The reductive potential was determined spectrophotometrically using a standard procedure [20]. Different concentration of the extract was mixed with 2.5 mL solution of 200mM phosphate buffer having pH 6.6 and 2.5mL solution of 1% potassium ferricyanide. The mixture was boiled for 20 min at 50°C. After incubation, 2.5 ml of 10% TCA was added to the mixture. The mixture was centrifuged for 10 min at 6500 rpm. After centrifugation, a 5mL solution of the upper layer was taken and mixed with 5ml of distilled water and 1ml of 0.1% ferric chloride. The absorbance of the solution was read spectrometrically at 700nm. Quercetin was used as a reference standard.

Total Antioxidant Activity: Total antioxidant activity was calculated by phosphomolybdenum method [21]. For determining total antioxidant activity, 0.3mL sample (1mg/mL concentration) was mixed with 3mL reagent solution containing 0.6 mL H_2SO_4, 28mM Sodium phosphate (1.2mL) and 4mM Ammonium molybdate (1.2 mL). The test tube containing reaction mixture was incubated at 95°C for 90 min. After the incubation, the solution was cooled at room temperature. Later, the absorbance was measured spectrometrically at 695nm against the blank. Ascorbic acid was used as the reference standard.

RESULTS AND DISCUSSION:

Yield of Extraction: The extraction of *S. aqueum* leaf powder using methanol as solvent system revealed a 65.62% yield.

Preliminary Phytochemical Screening: The result of preliminary phytochemical screening in methanolic leaf extract of *S. aqueum* showed the presence of several groups of secondary metabolic compounds, and the results are given in **Table 1**.

TABLE 1: PRELIMINARY PHYTOCHEMICAL SCREENING IN METHANOLIC EXTRACT OF *SYZYGIUM AQUEUM* LEAF

S. no.	Secondary metabolic constituents	Presence / Absence
1	Alkaloid	-
2	Cardiac glycosides	-
3	Coumarin glycosides	-
4	Saponin	+
5	Phenolic compound	+++
6	Flavonoids	++
7	Tannin	+
8	Carotenoids	+
9	Coumarin	-
11	Anthocyanin	-
12	Leucoanthocyanin	-
13	Quinone	-
14	Anthraquinone	-
15	Lycopene	-
16	Emolins	-
17	Steroids	+
18	Terpenoids	+

'+++' High concentration, '++ 'intermediate concentration, '+ 'low concentration, '-' absent

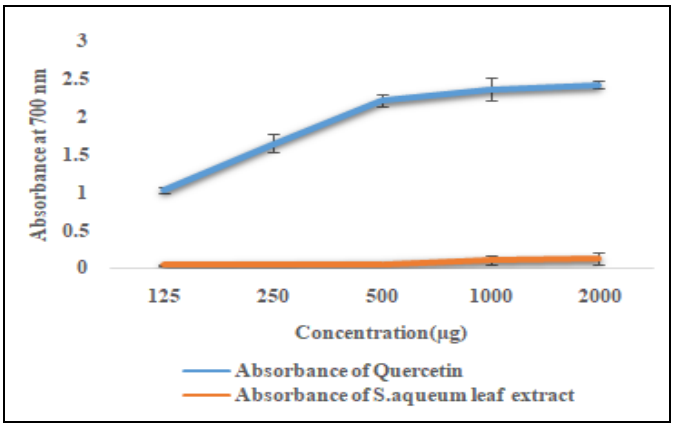
Phytochemicals are naturally occurring bioactive compounds accountable for different kinds of biological actions such as antioxidant action, hormonal action, and stimulation of enzymes, interference with DNA replication, antibacterial effect, and physical action [22]. The ultimate aim of phytochemical research is the invention of new bioactive compounds. In the present study, preliminary phytochemical screening and antioxidant activity in leaf extract of *S. aqueum* were determined as an initial step towards the investigation regarding the presence of different bioactive compounds. The result of preliminary phytochemical screening in leaf extract of *S. aqueum* revealed the presence of different bioactive phytochemicals such as phenolics, flavonoids, tannins, steroids, and terpenoids. Most of these compounds show immense therapeutic potential. Previous works regarding phytochemical screening in the genus *Syzygium* showed the presence of different categories of phytochemicals, which imparted better bioactivity to different members of this genus [23-24].

Quantification: The quantification of total phenol and flavonoid in leaf extract *S. aqueum* showed a good amount of total phenol (62.89 ± 1.48% GAE) and flavonoid content (15.05 ± 0.36% QE). Phenolic compounds are often important due to their high antioxidant, antiviral, anti-inflammatory, anti-cancer, and antidiabetic activity [25]. Antioxidants protect cells from oxidative stress-induced damages [26]. The present study gave evidence of high phenolic content in leaves of *S. aqueum*. High phenol content can act as a reducing agent, and also it imparts good antioxidant activity to the plant extract [27]. Many previous studies related to the correlation between antioxidant activity and total phenolic content revealed a positive correlation between them [28]. The previous study regarding antioxidant activity of the genus *Syzygium* suggested that the genus contain variety of bioactive phytochemicals, including phenolics and flavonoid compounds with good antioxidant activity [29-33].

Antioxidant Assay: The result of the different anti-oxidant analysis revealed the potential use of methanolic leaf extract of *S. aqueum* as a strong antioxidant agent. The result of antioxidant activity and IC_{50} of both standard and sample of each assay were represented in **Table 2** and **3**, **Graph 1**.

TABLE 2: RESULT OF ANTIOXIDANT ACTIVITY

S. no.	Antioxidant Assay	IC_{50} of Standard (µg/mL)	IC_{50} of Sample (µg/mL)
1	DPPH Assay	14.57±0.94	25.76±1.21
2	ABTS Assay	278.11±1.34	2077.18±2.97
3	Hydroxyl radical scavenging Assay	700.26±0.81	1512.20±3.01
4	Hydrogen peroxide radical scavenging Assay	424.10±1.07	7516.11±4.14
5	Nitric oxide radical scavenging Assay	170.20±1.65	1304.99±2.17
6	Superoxide radical scavenging Assay	281.14±1.02	1324.31±2.34



GRAPH 1: RESULT OF REDUCING POWER ASSAY

TABLE 3: TOTAL ANTIOXIDANT ACTIVITY

S. no.	Antioxidant assay	Activity of sample (mg% GAE)
1	Total Antioxidant activity	448.09±12.4

Natural antioxidants are important to maintain the health of living organisms. They function as an effective remedy to remove free radicals produced through normal cellular activity and from environmental stress [34]. Antioxidants reduce the harmful effect of these free radicals or Reactive Oxygen Species (ROS) [35]. DPPH scavenging assays has been widely used to determine the free radical activity of different substances [36]. This *in-vitro* antioxidant assay is considered highly reliable and useful to predict the antioxidant activity of substance within a short time. The mechanism behind the DPPH assay depends on the reduction of alcoholic DPPH solution in the presence of a hydrogen donating antioxidant due to the formation of a non-radical form of DPPH-H by the reaction.

DPPH reagents can readily release free radical in solution and form a violet color. Various antioxidant compounds present in the plant extract is able to scavenge released free radicals in solution, consequently, the violet color of the solution diminishes. Hence, the decrease in coloration is directly proportional to the free radical scavenging activity of the plant extract[35-38]. In the present study, the analysis of DPPH scavenging activity of leaf extract of *S. aqueum* revealed that the IC_{50} of standard (14.57±0.94 µg/mL) was slightly lower than the sample (25.76±1.21 µg/mL). The IC_{50} value of the leaf extract being in close range to the standard indicate its effectiveness in scavenging free radicals.

Test sample to be added in ABTS assay after ABTS cation has been generated by oxidation [39-40]. The amount of $ABTS^{\bullet+}$ remaining after reaction with antioxidants in the sample is measured and was expressed as Gallic Acid Equivalent (GAE). In this assay compared to standard (278.11 ± 1.34 µg/mL), the ABTS cation free radical scavenging activity of extract (2077.18 ± 2.97µg/mL) was less. However, the leaf extract demonstrated superoxide radical scavenging activity. Superoxides are weak radicals that often produce powerful hydrogen peroxide and singlet oxygen, both of which provide oxidative stress [41]. In a biochemical system, superoxide radicals and H_2O_2 react to form hydroxyl free radicals. These hydroxyl free radicals can attack and destroy almost all known biomolecules. Overproduction of superoxide anion radical contributes to certain redox imbalance, which is often associated with harmful physiological consequences. Superoxide anions are generated in the riboflavin-NADH system by the oxidation of NADH and assayed by the reduction of NBT resulting in the formation of blue formazan product [42]. In the present study, superoxide radical scavenging activity of extract showed an IC_{50} of 1324.31 ± 2.34µg/mL concentration compared to standard (281.14 ± 1.02 µg/mL).

Hydroxyl free radicals are potent ROS in the living system which reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids and cause damage to cells [43]. The result of hydroxyl radical scavenging assay showed that *S. aqueum* leaf extract (700.26 ± 0.81µg/mL) proved effective in scavenging hydroxyl radical compared to standard (424.10 ± 1.07µg/mL). But the hydrogen peroxide scavenging activity of leaf extract (7516.11± 4.14 µg/mL) was comparatively less than the standard (424.10 ± 1.07µg/mL). The hydrogen peroxide free radical scavenging ability of extract can reduce the DNA damage [44].

The result of Nitric oxide scavenging activity of extract proved that the methanolic leaf extract of *S. aqueum* (1304.99 ± 2.17 µg/mL) was less effective to scavenge Nitric oxide free radicals than the standard (170.20 ± 1.65 µg/mL). Nitric oxide (NO) has also been involved in several biological functions, including neurotransmission, vascular homeostasis, antimicrobial, and antitumor activities.

Despite the possible beneficial effects of nitric oxide, contribution to oxidative damage is also reported [46]. This oxidative damage is happened due to the fact that NO can react with superoxide to form the peroxynitrite anion, which is a potent oxidant that can decompose to produce OH and NO. The procedure is based on the principle that, at physiological pH, sodium nitroprusside in an aqueous solution spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions. A large amount of nitric oxide may lead to tissue damage, so the ability to extract to show nitric oxide scavenging activity is very much influenced the rate of tissue damage [47].

The reducing power capacity of plant extract was evaluated by monitoring the transformation of Fe^{3+} to Fe^{2+} in the presence of methanolic leaf extract. This method is based on the principle that an increase in absorbance of the reaction mixture indicates an increase in antioxidant activity of plant extract. The result revealed that the reducing power of leaf extract was less than the standard. The total antioxidant assay is based on the reduction of Mo (VI) to Mo (V) by the sample and subsequent formation of a green phosphate Mo (V) complex at acidic pH [48]. The total antioxidant activity analysis of plant extract also revealed good antioxidant activity (448.09±12.4 mg% GAE).

CONCLUSION: Bioactive phytochemicals present in plants are useful to human for curing various

ailments caused by dysfunction of biochemical reactions that usually take place in our body as part of metabolism. In the present study, the bioactive phytochemicals in the methanolic leaf extract of *S. aqueum* revealed the presence of bioactive secondary metabolites such as phenolic compounds, flavonoids, tannins, steroids and terpenoids. The quantification result of *S. aqueum* leaf extract also showed the presence of a good quantity of phenolic compounds and flavonoids. The secondary metabolic compounds present in the leaf extract of *S. aqueum* provides good antioxidant activity.

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CONFLICTS OF INTEREST: Nil

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