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Study of antimicrobial, antioxidant, Anti-inflammatory activities and phytochemical analysis of cooked and uncooked different spinach leaves

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Abstract

Spinach is a green leaf that grows in most parts of the world. In this paper a study has been carried on antimicrobial, antioxidant and anti-inflammatory activities and phytochemical analysis of cooked and uncooked spinach leaves. The Phytochemical analysis of carbohydrates, flavonoids, proteins, amino acids, steroids, tannins, anthroquinones, coumarin, saponins was carried out and this showed the presence of many bioactive compounds. The maximum total phenol content was found in cooked leaves of *Alternanthera sessilis* of $113.75 \pm 0.05 \mu\text{g GAE/mg}$ dry extract followed by uncooked leaves of *Tropical amaranth* of $86.25 \pm 0.14 \mu\text{g GAE/mg}$ dry extract. Spinach leaves showed effective and determinative antimicrobial activity against *Staphylococcus aureus*. The antioxidant activity showed the effective scavenging activity in cooked leaves of *Moringa oleifera* of $2.38 \pm 0.21 \mu\text{g/ml}$ and poor scavenging activity was found in uncooked leaves of *Alternanthera sessilis* of $38.79 \pm 0.33 \mu\text{g/ml}$. The anti-inflammatory activity showed maximum inhibition in cooked leaves of *Moringa oleifera* of $51.38 \pm 0.28 \mu\text{g/ml}$ and minimum inhibition in *Alternanthera sessilis* of $8.45 \pm 0.24 \mu\text{g/ml}$. These activities are useful for treating diseases and extracting drugs from spinach leaves.

Keywords: Spinacia oleracea, Alternanthera sessilis, Moringa oleifera, Tropical amaranth, Staphylococcus aureus

1. Introduction

Spinach is a green leaf vegetable and is grown in most parts of the world. Spinach leaves are the best source of dietary magnesium. They help in energy metabolism, maintaining muscle, nerve function, maintaining heart rhythm, a healthy immune system and blood pressure (Farah *et al.*, 2012, Subhash *et al.*, 2010) ^[1, 2]. Spinach leaves contain vitamins, minerals, antioxidants compounds and exhibit antimicrobial, anti-inflammatory activities (Merina & Souvik, 2013) ^[3]. In this study, four different types of spinach leaves were used *Spinacia oleracea*, *Alternanthera sessilis*, *Moringa oleifera* and *Tropical amaranth*. *Spinacia oleracea* belongs to the family of Chenopodiaceae and is useful in treating diseases of blood and brain, asthma, leprosy, biliousness

It is used in the treatment of urinary calculi, difficulty in breathing, inflammation of liver and jaundice and has poglycemic properties (Namrata *et al.*, 2016) ^[4]. *Alternanthera sessilis* belongs to the family of Amaranthaceae. It is a perennial herb. The branches grow from the root up to 50 cm long. *Alternanthera sessilis* belongs to the family of Amaranthaceae. It is perennial herb. The branches row from the root up to 50 cm long. This plant found in damp places, wet headlands, roadsides, sometimes as weed in plantations and it is useful for treatment of gastrointestinal problem (Archana *et al.*, 2011) ^[5]. *Moringa oleifera*, which belongs to the family of Moringaceae grows widely in many tropical and subtropical countries. It is used as a traditional medicinal source. All the parts of the *Moringa* tree such as pods, seeds, and leaves are edible. It is used for the treatment of many diseases. It offers essential and disease preventing nutrients to humans and it is called a 'miracle vegetable' (Fahmy *et al.*, 2015) ^[6]. *Tropical amaranth* belongs to the family of Amaranthaceae. *Amaranth* called as "never-fading flower". It is used to cure for diarrhoea, dysentery, excessive menstrual flow, ulcers and intestinal haemorrhaging (Saud Asif *et al.*, 2013) ^[7]. Spinach leaves extract exhibits antimicrobial activity and antioxidant activity, it inhibits the oxidative damage to target molecules and has characteristics to trap the free radicals and contains compounds like phenolic acids, polyphenols, carotinoids flavonoids, scavenge free radicals such as peroxide, hydroperoxide and inhibits the oxidative process that leads to degenerative disease. So spinach leaves are considered to have a good antioxidant potential (Akhilesh *et al.*, 2010) ^[8]. Spinach

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Leaves have Anti-inflammatory property, a substance that reduces an inflammation or swelling and many phytochemicals are present in spinach leaves extract (Godhandaraman *et al.*, 2016; Jamuna Senguttuvan *et al.*, 2014) [9, 10]. The main objective of this study is extraction and analysis of phytochemical, antimicrobial, antioxidant and anti-inflammatory activities of cooked and uncooked spinach leaves.

2. Materials and Methods

2.1. Plant materials

Fresh leaves of *Spinacia oleracea*, *Alternanthera sessilis*, *Moringa oleifera* and *Tropical amaranth* were collected from a local market in Chennai, Tamil Nadu.

2.2. Preparation of Extracts

100 g of leaves sample was taken individually and cut into small pieces. 50 g of samples were cooked at 45°C for 10 min and 50 g of samples were uncooked and dried in shade for a week. The samples were powdered using the blender. 10 g of samples were extracted with 100 ml of methanol individually and incubated in a rotary shaker for 24 hr, the extracts were filtered using Whatman filter paper no.4. The collected solvents were dried by using drying oven incubator at 60°C to form dry powder. The dried mass was dissolved to get a 10 mg/ml concentration of extract.

2.3. Test microorganisms

The test microorganism used for the antimicrobial activity was *Staphylococcus aureus* obtained from the Apex Biotechnology Research and Training Institute, Chennai, Tamil Nadu

2.4. Qualitative Phytochemical analysis

Qualitative photochemical test was carried out with the methanol extracts of cooked and uncooked spinach leaves (Sofowora, 1993; Trease & Evans 2002) [16, 17].

2.4.1. Test for carbohydrates

1 ml of extract is mixed with a few drops of Benedict's reagent and boiled in water bath for few minutes. Formation of red or pink colour shows presence of carbohydrates.

2.4.2. Test for anthraquinones

About 0.1 g of each extract to be tested is shaken with 5 ml of benzene and then filtered. 3 ml of 10% ammonia solution is added to a filtrate and shaken. Appearance of pink, red and violet colour in lower phase shows the presence of anthraquinones.

2.4.3. Test for flavonoids

About 0.1 g of leaf extract is boiled with distilled water and then filtered. 2 ml of filtrate with few drops of 10% ferric chloride solution is added. Green, blue or violet colour shows the presence of flavonoids.

2.4.4. Test for proteins

0.2 g of sample added with 1ml of distilled water is treated with 10% NaOH solution and two drops of 0.1% CuSO₄ solution. Formation of pink /violet colour shows the presence of proteins.

2.4.5. Test for free amino acids

0.5 ml of test solution is boiled with 0.2% solution of a ninhydrin. Formation of purple colour shows the presence of free amino acid.

2.4.6. Test for coumarin

To 1 ml of test solution, a few drops of alcoholic NaOH is added. Appearance of yellow colour indicates the presence of coumarin.

2.4.7. Test for saponins

200 mg of extract is boiled with 5 ml of distilled water and filtered. To the filtrate, 3 ml of distilled water is added further and shaken vigorously for 5 min. Frothing which persisted on warming is taken as indicator of the presence of saponins.

2.4.8. Test for steroids

To 0.2 g of each portion, 2 ml of acetic acid is added; the solution is cooled well in ice followed by addition of conc. H₂SO₄. Colour change from violet to blue or bluish green indicated the presence of a steroidal ring i.e., aglycone portion of cardiac glycoside.

2.4.9. Test for tannins

About 0.1 g of each portion is stirred with about 2 ml of distilled water and filtered. Few drops of 1% ferric chloride solution is added to 2 ml of filtrate. Formation of a blue-black, green or blue-green precipitate indicates the presence of tannins.

2.5. Estimation of total phenol

The solvent extracts used for the determination of total phenol were done spectrophotometrically according to Folin-Ciocalteu colorimetric method. Each extract (200 µl) was introduced into screw cap test tubes and 1 ml of Folin-Ciocalteu reagent (1:1 with water) and 1 ml of sodium carbonate (7.5%) were added. The tubes were vortexed and incubated for 2 hr and the absorbance was read at 726 nm using a UV- Vis spectrophotometer. The total phenol content was expressed in Gallic Acid Equivalent (GAE) mg/g dry material (Singleton & Rossi, 1965; Lin & Tang, 2007) [12, 13]. Total phenol was calculated by using the equation

$$\text{Total phenol} = (\text{Control OD} - \text{Test OD}) \div \text{Control OD} \times 100$$

2.6. Antimicrobial activity

The antimicrobial activity was carried out by agar well diffusion method to detect the presence of antimicrobial activity in the samples extract. Sterile nutrient agar was prepared and poured into sterile petri plates. The nutrient agar was solidified and a well was prepared in the plates with the help of a borer. The sterile swab was used to distribute the bacterial culture (*S. aureus*) evenly over the surface of the nutrient agar plates. Then the plates were allowed to dry for 10 min and the test samples were charged into the well at different concentrations (25, 50, 75, 100 µg/ml). The antibiotic Streptomycin was introduced into the centre of the well for control. Then the plates were labelled and incubated overnight at 37°C. Microbial growth was determined by measuring the diameter of zone of inhibition (Merina & Souvik, 2013; Namrata *et al.*, 2016) [3, 4].

2.7. Antioxidant activity

The antioxidant activity was studied using the DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay. 2 ml of 6×10⁻⁵ M methanol solution of DPPH was added to a 50 µl of sample at a concentration of 10 mg/ml. The samples were incubated for 16 min at room temperature in a dark place and DPPH served as an absorbance of control and absorbance was read at 515nm using a UV- Vis

spectrophotometer. The scavenging effect (decrease of absorbance at 515 nm) was the percentage of DPPH radical scavenging ability of the samples calculated from the absorbance value at the end of 16 min (Von Gadow *et al.*, 1997) (Yen & Duh, 1994) [14, 15]. The percentage inhibition of DPPH radical of the samples was calculated by using the equation

$$IP = (Ac(0) - Aa(t)) \div A(0) \times 100$$

Where, Ac (0) is the absorbance of the control at t = 0 min and Aa (t) is the absorbance of the antioxidants at t = 16 min.

2.8. Anti-inflammatory Activity

The anti-inflammatory activity was studied by using the inhibition of protein denaturation method. The reaction mixture consists of a 100 ml egg albumin (white yolk from fresh hen's egg) 9.9 ml of double distilled water, 20 µl of concentration HCl and 25 µl of sample extracts. Reaction mixture without the test samples served as a blank. The samples were incubated at 37° C for 20 min and 57° C for 30 min. After cooling, the absorbance of samples was measured at 660 nm in a UV-Vis spectrophotometer (Godhandaraman *et al.*, 2016) [9]. The percentage inhibition of protein denaturation was calculated by using the equation

$$IP = (Ac(0) - Aa(t)) \div A(0) \times 100$$

Where, Ac (0) is the absorbance of the control at t = 0 and Aa (t) is the absorbance of the sample.

3. Results and Discussion

3.1. Phytochemical analysis

The qualitative phytochemical analysis for cooked and uncooked spinach leaves revealed the presence of carbohydrates, flavonoids, proteins, amino acids, steroids, tannins and absence of anthraquinones, coumarin, saponins in the methanol extracts of *Spinacia oleracea*, *Alternanthera sessilis*, *Moringa oleifera*, *Tropical amaranth* and result are shown in Table 1. Phytochemical analysed of the plant extracts revealed the presence of constituents which showed medicinal and physiological activities (Safowora, 1993) [16]. In the previous study, methanolic extracts of the leaves *Spinacia oleracea* showed the presence of carbohydrates, proteins, amino acids, steroids, terpenoids, glycosides, alkaloids, tannins and other phenol compounds (Namrata *et al.*, 2016) [4]. In the previous study, ethanolic extracts of leaves *Alternanthera sessilis* showed the presence of alkaloids, flavonoids, aminoacids, carbohydrates, phenols, steroids, terpenoids, saponins and glycosides and absence of tannins (Sivakumar & Sunmathi, 2016) [18]. In the previous study, *Moringa oleifera* showed presence of alkaloids, flavonoids, carbohydrates, glycosides, proteins, saponins, tannins and terpenoids in ethanolic solvent extracts (Pinal Patel *et al.*, 2014) [19].

Table 1: Qualitative Phytochemical Analysis of cooked and uncooked spinach leaves

Constituents	Cooked leaves				Uncooked leaves			
	A	B	C	D	A	B	C	D
Carbohydrates	+	+	+	+	+	+	+	+
Anthraquinones	-	-	-	-	-	-	-	-
Flavonoids	+	+	+	+	+	+	+	+
Amino acids	+	+	+	+	+	+	+	+
Proteins	+	+	+	+	+	+	+	+
Coumarin	-	-	-	-	-	-	-	-
Saponins	-	-	-	-	-	-	-	-
Tannins	+	+	+	+	+	+	+	+
Steroids	+	+	+	+	+	+	+	+

Note: A- *Spinacia oleracea*; B- *Alternanthera sessilis*; C- *Moringa oleifera*; D- *Tropical amaranth* ; + ve indicates presence, whereas - ve indicates absence

3.2. Estimation of total phenol content

The total phenol content of methanol extract for cooked and uncooked spinach leaves was tested using Folin-Ciocalteu colorimetric method and it showed significant amount of phenols in all the tested extracts. About 4 mg of extract was used to determine the total phenol content and calculated by using the equation in 2.5. The higher total phenol content was found in cooked leaves extract of *Alternanthera sessilis* showed 113.75 ± 0.055 µg GAE/mg dry extract and lower content was found in uncooked leaves of *Tropical amaranth* showed 86.25 ± 0.147 µg GAE/mg dry extract and the result as shown in Table 2. The methanol extracts of cooked and uncooked spinach leaves showed the presence of phenol content and is used as an intermediates in industries (Namrata *et al.*, 2016). [4]. The phenol compounds are one of the largest and high pervasive groups of plant extracts (Singh *et al.*, 2007) [25].

3.3. Antimicrobial activity

The antimicrobial activity of cooked and uncooked leaves extract was different in intensity and specificity. The extracts from cooked and uncooked leaves of *Spinacia oleracea*, *Alternanthera sessilis*, *Moringa oleifera*, *Tropical amaranth* showed the effective and determinative antimicrobial activity against the bacterial strain of *Staphylococcus aureus*. 75 and 100 mg/ml showed a similar result of zone of inhibition. In the previous study, the results appeared to be in refutation with an earlier report that all green vegetables including spinach have no antibacterial activity against *S. epidermidis* and *K. Pneumonia* (Lee YL *et al.*, 2003) [20]. Finding of new antimicrobials is very important at present considering the higher levels of antibiotic resistance among pathogenic bacteria (Hatha *et al.*, 1995; Srinivasan *et al.*, 2001) [21, 22]. The positive control of streptomycin showed the effective activity against *Staphylococcus aureus*. The higher zone of inhibition was found in *Spinacia oleracea* and *Moringa oleifera* of cooked extract 6 mm and the result of zone of inhibition are shown in Table 3 & 4 and Fig. 1 & 2.

Table 2: Total phenol content for cooked and uncooked spinach leaves

Plant	Cooked leaves	Uncooked leaves
<i>S. oleracea</i>	100.416 ± 0.492	98.33 ± 0.205
<i>A. sessilis</i>	113.75 ± 0.055	84.58 ± 0.409
<i>M. oleifera</i>	97.5 ± 0.329	87.68 ± 0.364
<i>T. amaranth</i>	92.5 ± 0.262	86.25 ± 0.147

Note: Values expressed in mean ± standard deviation.

Table 3: Antimicrobial activity of cooked samples extract

Plant	Concentration (µg/ml)				Control
	25	50	75	100	
<i>S. oleracea</i>	0	0	6	4	9
<i>A. sessilis</i>	0	0	2	1.5	7
<i>M. oleifera</i>	0	1.5	4	6	9.5
<i>T. amaranth</i>	0	2	3.5	5	8

Note: Values expressed in mm.

Table 4: Antimicrobial activity of uncooked samples extract

Plant	Concentration (µg/ml)				Control
	25	50	75	100	
<i>S. oleracea</i>	0	0	3	4	7
<i>A. sessilis</i>	0	0	1	2.5	9.5
<i>M. oleifera</i>	0	1	3.5	4	9
<i>T. amaranth</i>	0	1.5	2.5	4	8.5

Note: Values expressed in mm

3.4. Antioxidant activity

The DPPH radical scavenging activity results of cooked and uncooked leaves are shown in Table 5 and calculated by using equation in 2.7. The methanol extracts of cooked and uncooked leaves exhibit antioxidant activity. The observed reduction of DPPH by the extract was either due to the transfer of a hydrogen atom or the transfer of an electron. Phenol compounds are also effective hydrogen donors, which make them good antioxidants (Michalak, 2006) [23]. Antioxidants come from plants in the form of phenol compounds such as flavonoid, phenol acids, tocopherols (Ali et al., 2008) [26]. DPPH is a -known radical scavenger, the rate reduction of a chemical reaction based upon addition of DPPH was used as an indicator of the radical nature of that reaction. Because of strong absorption, the DPPH radical has a deep violet colour in the solution, and it becomes colourless or pale yellow when neutralised.

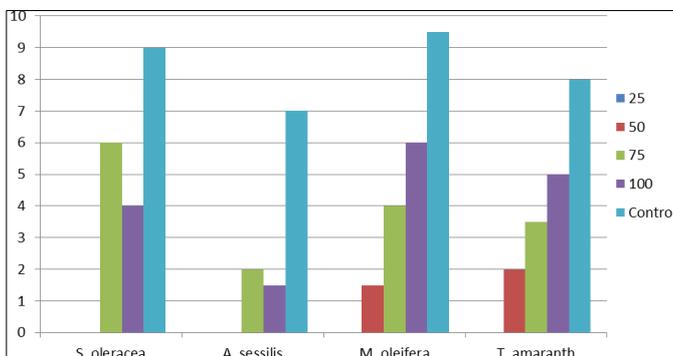


Fig 1. Antimicrobial activity of cooked sample extract against *S. aureus*.

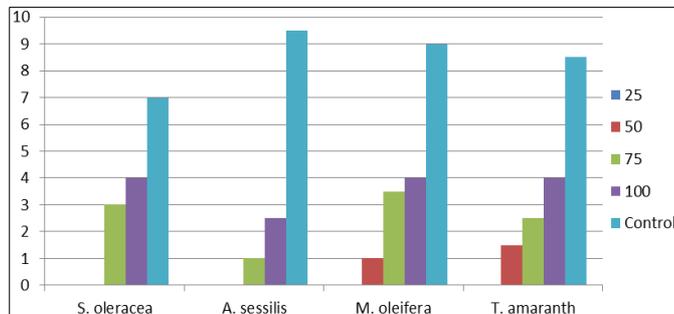


Fig 2. Antimicrobial activity of uncooked sample extract against *S. aureus*

Table 5: Antioxidant activity of cooked and uncooked samples extract

Plant	Cooked	Uncooked
<i>S. oleracea</i>	38.15 ± 0.209	10.17 ± 0.254
<i>A. sessilis</i>	17.48 ± 0.219	38.79 ± 0.33
<i>M. oleifera</i>	2.38 ± 0.213	11.44 ± 0.183
<i>T. amaranth</i>	17.32 ± 0.265	9.69 ± 0.206

Note: Values expressed in mean ± standard deviation.

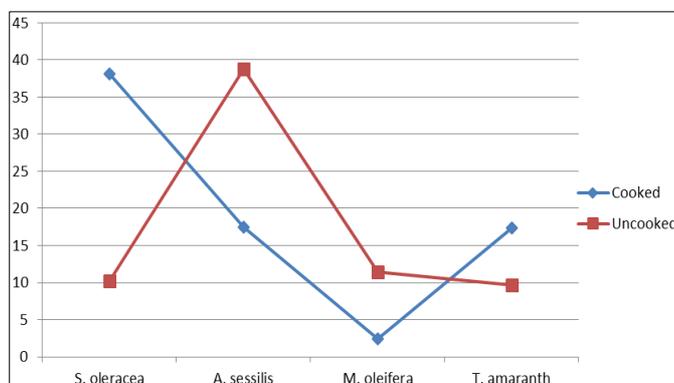


Fig 3. Antioxidant activity of cooked and uncooked samples extract

In the previous study, of the antioxidant activity fresh and frozen spinach extracts were examined and sample showed higher content of frozen spinach than for fresh spinach (Magdalena et al., 2013) [24]. In this study, the cooked leaves extract *Moringa oleifera* showed the lower radical scavenging activity in the methanol extracts 2.38 ± 0.213 µg/ml and *T. amaranth* showed higher value 38.15 ± 0.209 µg/ml which indicates poor scavenging activity. In uncooked methanol extract of *Tropical amaranth* showed a lower radical scavenging activity of 9.69 ± 0.206 µg/ml and *A. sessilis* showed higher value 38.79 ± 0.33 µg/ml which indicates poor scavenging activity.

Table 6: Anti-inflammatory activity of cooked and uncooked different spinach leaves

Plant	Cooked	Uncooked
<i>S. oleracea</i>	26.58 ± 0.261	44.692 ± 0.340
<i>A. sessilis</i>	8.475 ± 0.244	38.89 ± 0.168
<i>M. oleifera</i>	51.38 ± 0.285	49.59 ± 0.241
<i>T. amaranth</i>	43.61 ± 0.150	44.24 ± 0.521

Note: Values expressed in mean ± standard deviation.

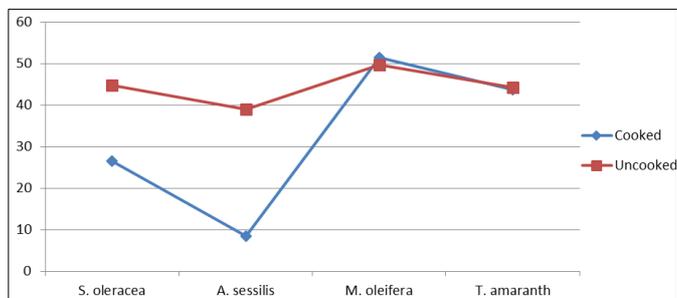


Fig 4: Anti-inflammatory activity of cooked and uncooked sample extracts

3.5. Anti-inflammatory activity

Protein denaturation is a decrease of biological properties of protein molecules. Denaturation is responsible for the cause of inflammation conditions like rheumatoid arthritis, diabetes, cancer and prevention of protein denaturation, may also help in preventing inflammatory conditions. In the previous study, the maximum inhibition of 70.43% was observed in stem extract of *P. murex* at a concentration of 1000 µg/ml and the minimum inhibition of 31.51% was shown in the leaf extract at a concentration of 200 µg/ml (God handaraman *et al.*, 2016) [9]. The present study shows the anti-inflammatory activity of methanol extract of cooked and uncooked spinach leaves on inhibiting denaturation of proteins as shown in the Table 6 and calculated by using equation in 2.8. The maximum inhibition 51.38 ± 0.285 µg/ml was observed in cooked leaves of *Moringa oleifera* and the minimum inhibition 8.45 ± 0.244 µg/ml was observed in cooked leaves of *A. sessilis* at a concentration of 20 mg/ml. On comparison, the cooked spinach leaves extract showed maximum inhibition than uncooked spinach leaves extract.

4. Conclusion

The present study reveals the presence of many phytochemical compounds like carbohydrates, flavonoids, proteins, amino acids, tannins and steroids in cooked and uncooked spinach leaves. It showed the effective and determinative antimicrobial, antioxidant, anti-inflammatory activities. Cooked spinach leaves shows better results than uncooked spinach leaves extracts in all the activities because cooked leaves absorbs higher levels of energy. Spinach leaves are good for dietary, energy metabolism, nerve function, maintaining blood pressure, heart rhythm etc and in future it may help to extract a drug to cure diseases.

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