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Anant Babu Marahatta
Department of Chemistry, Amrit Science College, Tribhuvan University, Kathmandu, Nepal

Aashish Aryal
Central Department of Chemistry, Tribhuvan University, Kathmandu, Nepal

Ram Chandra Basnyat
Central Department of Chemistry, Tribhuvan University, Kathmandu, Nepal

Correspondence
Anant Babu Marahatta
Department of Chemistry, Amrit Science College, Tribhuvan University, Kathmandu, Nepal

The phytochemical and nutritional analysis and biological activity of *Annona squamosa* Linn.

Anant Babu Marahatta, Aashish Aryal and Ram Chandra Basnyat

Abstract
Nepal, being a land of diverse topography with different weather condition, the types of secondary metabolites/phytoconstituents of the locally grown *Annona squamosa* plant (*SARIFA*, सरीफा in Nepalese language) could be diverse. That is why, study of the phytochemical and nutrirional analysis and biological activity of such plant grown in the local weather is extremely essential. As this plant is very popular due to its wide application as traditional medicine from ancient times to this day, we have chosen its type grown in the subtropical region of Nepal as experimental specimen and extracted the contents of its leaves and bark in methanol and hexane solvents separately by Soxhlet extraction method. The phytochemical screening process has unveiled alkaloids, polyphenols, flavonoids, and tannins in either of these extracts. The total phenolic and flavonoid content for the methanol extract of leaves and bark are estimated as 217.82 and 160.48 mg/g GAE and 66.92 and 76.50 mg/g QE respectively. In DPPH free radical scavenging test, the methanol and hexane extract of leaves shows the highest and second highest antioxidant activities. In α-amylase inhibition assay, the methanol extract of bark shows considerable amount of antidiabetic activities. The significant ZOI, MIC and MBC values for the methanol extracts of leaves and bark confirm their abilities to cure the diseases caused by *Escherichia coli, Klebsiella pneumonia* and *Staphylococcus aureus* bacteria. The major chemical compounds: Palmitic acid and Geranyl linalool <E, E> in leaves and Isopimarol and Sandaracopimarinol in bark are also predicted by GC/MS analysis.

Keywords: *Annona squamosa* (*SARIFA*, सरीफा), Traditional medicine, Hexane and Methanol extracts, Antimicrobial, Antidiabetic and Antioxidant

1. Introduction
Natural resources are those materials which occur naturally and are provided by the Earth, which means that humans cannot make them. Instead, they can be used and modified in ways that are beneficial to humans. Nature stands as an infinite natural resources including varieties of plants producing chemically distinct natural products such as biotic (e.g. wood, silk), bio-based (e.g. bioplastics, cornstarch), bodily fluids (e.g. plant exudates), and other natural materials (e.g. soil, coal) [1]. Around the world, the natural products continue provide an alternative to modern medicine in drug discovery [1, 2]. Many scientific studies and investigations about the natural products have revealed the science of organic chemistry that ultimately leads to synthetic organic chemistry where scientists create organic molecules in the laboratory, and semi-synthetic organic chemistry where scientists modify existing natural products to improve or alter their activities. More specifically, the role and contributions of natural products chemistry and research dealing with the natural products in the advancement of physical and biological sciences are indispensable.

Plants are those natural resources which are capable of synthesizing large varieties of organic compounds of very unique and complex structures that are categorized into primary metabolites (hereafter, PMs) and secondary metabolites (hereafter, SMs). The PMs have an intrinsic functions that are associated with essential cellular functions such as nutrition assimilation, energy production, growth, and development and in contrast, the SMs have an extrinsic function that play an important role in plant defense against herbivory and other interspecies defenses. Thus, former is essential for the survival of the plants and the latter increases their competitiveness within the environment. The steroids, terpenoids, flavonoids, alkaloids, quinones, polyphenols etc. are SMs biosynthesized from PMs [3]. Since ancient times and up to this day, mankind has been using SMs as the resources for medicines, flavorings, fragrances, pesticides, poisons, stimulants, dyes, perfumery and countless more purposes. Because of facing many difficulties for frequent extraction but having tremendous potentialities for developing wide spectrum drugs, SMs have been important targets for bioengineering [4-7].

The natural products limiting to SMs are most commonly used in the fields of medicinal chemistry and pharmacognosy in which the study and use of natural products in medicine is a principal objective. The SMs are already found to have potential activities in curing various types of diseases. Such plants with curative constituents for number of listed diseases are termed as Medicinal plants [8]. The Earth consists rich wealth of medicinal plants [9]. Approximately 85,000 plants have been found to have medicinal characteristics worldwide [10]. Out of them, Nepal hosts 1,624 species of medical and aromatic plants [11]. The Himalayas are more famous for medicinal plants which have been integral part of traditional medicine practices of indigenous Nepalese community. It supports Nepal, being one of the largest exporter of the medical herbs showing good reputation in international market for their medical benefits.

The plant we have chosen here to study the phytochemical and nutritional analysis and biological activity is most commonly known as *SARIFA*, *सरीफा* in Nepalese language. It belongs to the family Annonaceae: custard apple family that includes many flowering plants consisting trees, shrubs, or rarely lianas [12]. With 108 accepted genera and about 2400 known species, the Annonaceae family is the largest in the Magnoliales and mostly concentrated in the tropical and sub-tropical, with few species found in temperate regions. Several genera of this family produce edible fruits, most notably *Annona*, *Anonidium*, *Asimina*, *Rollinia*, and *Uvaria* [12, 13]. *SARIFA*, *सरीफा* has its type genus *Annona*. It is widely known by its scientific name *Annona squamosa* (hereafter, *A. squamosa* or AS) and common name sugar apple tree. In Nepal, it is mostly grown for its delicious fruits in the sub-tropical zones in areas below 1000m above mean sea level with the annual average temperature of above 20º C. It ranges from 10 to 20 ft in height with open crown of irregular branches as shown in chart 1a. Along the branch tips, opposite the leaves, the fragrant flowers are borne singly or in groups of 2 to 4 as shown clearly in chart 1b. The flowers are oblong, 2.5-3.8 cm long, never fully open, drooping stalks, and 3 fleshy outer petals, yellow-green on the outside and pale-yellow inside with a purple or dark-red spot at the base. The leaves shown in the chart 1c measure 2 to 6 inches in length and feature hairy petioles. These oblong leaves emit a strong fragrance when they are crushed. The compound fruit is nearly round, ovoid, or conical, 6-10 cm long composed of knobby segments (chart 1b). There may be a total of 20 to 38 or perhaps more seeds in an average sized fruit. Some trees, however, bear seedless fruits. A mature tree bears in an average 50 fruits [13, 14]. The fruits ripen very quickly at ambient temperatures within several days after maturation. Fruits softening, splitting and cracking are the major postharvest problems. The ripe sugar apple is usually broken open and the flesh segments enjoyed while the hard seeds are separated in the mouth and spat out. In the plane region of Nepal where weather is very hot, it is common that the flesh segment is pressed through a sieve to eliminate the seeds and is then added to ice cream or blended with milk to make a cool beverage.

The plant *A. squamosa* is known to show Antioxidant, Antidiabetic, Enzyme inhibition activity while the fruit is very popular due to low carbohydrate content. A large number of chemical compounds, including flavonoids, alkaloids, and acetogenins have been extracted from the seeds and many other parts of these plants. Besides these, the flavonoids and alkaloids contained in the leaves and bark have shown insecticidal properties [12]. The bark, leaves, and roots are used in the traditional medicines. Traditionally, it is used as an insecticidal, anti-tumor, anti-diabetic, anti-oxidant, anti-lipidemic, and anti-inflammatory agent which may be characterized due to the presence of the cyclic peptides. The seeds are reported to have anti-parasitic activities (against lice). The crushed leaves are applied on ulcers and wounds and a leaf decoction is taken in case of dysentery. Similarly, the crushed leaves have been using to revive a person who has fainted or to help overcome hysteria. The common people consumes a mixture of 4-5 newly grown leaves along with black pepper (*Piper nigrum*) for the management of diabetes. The bark decoction is given as a tonic and to halt diarrhea [15]. Because of such exceptional medicinal and nutritional values of *A. squamosa*, we selected this plant grown in the sub-tropical region of Nepal as experimental specimen and studied its phytochemical and nutrirional analysis and biological activity. Even thogh, many studies about the medicinal values of *A. squamosa* grown abroad have been reported [15-17], no concreat reserch works are performed so far concentrating on locally grown *A. squamosa* in Nepal weather. It is well known that the types of the natural products, their chemical constitunets and ultimately their use as traditional medicines rely on nature and natural climatic scenario: (temperature; air/wind; humidity; precipitation/raining and sunlight). Each of the descriptor plays a direct or indirect role in the buildup of secondary metabolites/phyto-constituents of plants [18]. Some descriptors even directly control the bioactivity of respective phyto-constituents. For example, a significant increase in temperature (as in summers) may lead to evaporation of volatile compounds like oils from the plants; heat strokes may cause volatile phyto-constituents to evaporate at a faster rate; a high rainfall helps to elevate production of phyto-constituents; certain components of sunlight such as UV-A and UV-B have been reported to be directly linked to the production of phenolics and tannins [18]. That is why, we though it would be indispensable to reveal the phytochemicals, antioxidants, antidiabetics, antibacterials, and percentage composition of nutrirional parameters of the locally grown *A. squamosa* plant. In order to accomplish these major objectives, we at first extracted the plant extracts and investigated thoroughly the phenolic content, flavonoid content, antidiabetic assay, antioxidant assay and antibacterial assay. The methodologies we employed here are already adopted in our previous study [19]. Additinally, we have unveiled the major chemical compounds present in the plant extracts by Gas chromatography-mass spectrometry (GC/MS) analysis. The paper is organised as follows: Materials and Methods, Results and Discissions and Conclusion.

2. Materials and Methods

2.1 Material

The fresh leaves and bark of locally grown *A. squamosa* were collected from the sub-tropical region: Adamghat, Pida – 07, Dhading (Province number 3), Nepal and the fruits were collected from the same place during ripening season (May/June). The taxonomic identification of the plants was carried out at Central Department of Botany, Tribhuvan University, Kirtipur by judging the preserved herbariums carefully.

2.2 Method

2.2.1 Extraction

The collected fresh leaves and bark of *A. squamosa* were washed with tap water to remove the contaminants. Then the

leaves and bark were shade dried and grounded into powder form in electric grinder and stored in clean plastic bag separately until further use. The phytochemicals present in the powdered leaves and barks were extracted by percolation method using Soxhlet extraction method. The powdered sample was taken in two separate thimbles of Soxhlet extractor and the setup was organized accordingly. The round bottom flask was filled with hexane up to its two-third and was adjusted to the extractor. Finally, the solvent was heated over the heating unit at the temperature of about 40ºC and the extraction process was allowed to run for about an hour. After the completion of the extraction process, the solvent with plant extract was subjected to concentration process using the rotary evaporator at 40ºC. Thus obtained hexane extract was dried over heating element and then stored in a beaker wrapped by aluminium foil until further use. Similarly, the methanol extract was also obtained by the similar process by heating the solvent at about 70ºC.

2.2.2 Phytochemical analysis
The phytochemicals present in different plant extracts were analyzed by following the protocol given by Ciulei I. (2003) [20].

2.2.3 Total phenolic content
The total phenolic content (TPC) in plant extract was analyzed by Folin-Ciocalteu colorimetric method based on oxidation-reduction reaction as described by Waterhouse [21]. Gallic acid is used as the standard as it is less expensive, purely available and more essential in any DPPH (1, 1 diphenyl-2-picryl hydrazyl) radical scavenging method.

2.2.4 Total flavonoid content
The total flavonoid content (TFC) of the plant extract was determined by aluminium chloride ($AlCl_3$) colorimetric Assay [22]. Quercetin, a polyphenol compound is used as a standard as it is very common to study the antioxidant activity in DPPH research.

2.2.5 Antioxidant assay
Antioxidant activity of different plant extracts was done by DPPH radical scavenging method as described by Blois [23].

2.2.6 Anti-diabetic assay
Anti-diabetic activity of plant extracts was determined from α-amylase inhibition assay [24].

2.2.7 Antibacterial assay
Inhibition of bacterial growth was tested by using agar well plate method and measured in the form of zone of inhibition (ZOI) as given by Dingel *et al.* [25]. The Antibacterial assay was performed at Gandaki Medical College, Pokhara, Nepal. Some snapshots of the specific tests are shown in chart 1d-1f.

2.2.8 Proximate analysis of nutritional value of fruit
The approximate analysis for the nutritional composition of the fruit of the *A. squamosa* was determined according to the protocol given by AOAC [26].

3. Results and Discussions
3.1 Phytochemical Assay
The micro-chemical analysis of crude extract of AS leaves and bark in each methanol and hexane solvent depicted the presence of following phytochemicals listed in Table 1. This qualitative phytochemical assay specifies that the leaves and bark contain a significant amount of alkaloid, flavonoids, phenolic, saponins, and tannins. As expected, the volatile oils are present in either of the two extracts of leaves.

Table 1: Phytochemical analysis of leaves and bark. Key: (+): Present (−): Absent

S.N.	Classes of phytochemicals	Hexane extract of leaves	Methanol extract of leaves	Hexane extract of bark	Methanol extract of bark
1.	Volatile Oils	+	+	−	−
2.	Alkaloids	−	+	−	+
3.	Terpenoids	+	−	+	−
4.	Coumarins	−	−	−	−
5.	Flavonoids	+	+	+	+
6.	Quinones	−	−	−	−
7.	Polyphenols	+	+	+	+
8.	Glycosides	−	+	−	+
9.	Carbohydrates	−	+	−	+
10.	Saponins	−	+	−	+
11.	Tannins	−	+	−	+

3.2 Total phenolic and flavonoid content
By using calibration curve and absorbance values (Figure 1) (triplicates of 1000 µg/mL), total phenolic contents (hereafter, TPCs) for methanol and hexane extracts of leaves and barks of *A. squamosa* are calculated. The TPCs for methanol extract of leaves and bark are calculated as 217.82 and 160.48 mg per gram Gallic acid equivalent (mg/g GAE) respectively. Similarly, TPCs for hexane extract of leaves and barks are calculated as 200.98 and 29.98 mg/g GAE respectively as shown in Figure 2. Additionally, by using calibration curve and absorbance values (Figure 3) (for triplicates of 1000 µg/mL), total flavonoid contents (hereafter, TFCs) for methanol extract of leaves and barks are also calculated. They are found to be 66.92 and 76.50 mg per gram quercetin equivalent (mg/g QE) respectively. The TFCs for hexane extract of leaves and barks are estimated as 27.96, and 23.48 mg/g QE respectively (Figure 4).

From the results obtained from TPC and TFC analysis, it is found that flavonoids show antioxidant activity by its scavenging property or chelating process. They are one of the many molecules that are used by cells for the protection against the harmful effects of reactive oxygen species. The phenolic compounds are class of antioxidants which acts as free radical terminator and possess scavenging ability due to their hydroxyl group. The methanol extract of AS leaves and bark contains the highest TPC: 217.82 mg/g GAE and TFC: 76.5 mg/g QE respectively than any other extracts prepared here, as shown in Figure 2 and Figure 4 respectively. As the phenolic compounds can reduce the risk of oxidative stress induced diseases and the flavonoids show higher antidiabetic

activity, the methanol extracts of leaves and bark could be utilized for medicinal purposes. The difference in phenolic and flavonoid content for the extracts may be attributed to the solvents used in extraction. The first solvent methanol is a polar compound due to the -OH group and the second solvent hexane is nonpolar because there are only carbon and hydrogen atoms. The polar substances cannot dissolve/mix with nonpolar substances. That is why, the more polar solvent methanol has more power for recovering polar polyphenols and flavonoids from the plant matrices. Thus, the methanol extracts in comparison to the hexane extracts contain high phenolic and flavonoid compounds.

3.3 Antioxidant activity

The antioxidant activity of methanol and hexane extracts of *A. squamosa* are studied by plotting% free radical scavenging vs concentration (Figure 5 and Figure 6) and the half maximal inhibitory concentration (hereafter, IC_{50}) values for respective extracts are calculated. Thus calculated IC_{50} value for different extracts are compared with IC_{50} value for standard solution: ascorbic acid. The IC_{50} value for ascorbic acid is calculated to be 38.44 µg/mL. The IC_{50} values for methanol and hexane extracts of leaves are found as 49.64 and 64.01 µg/mL and those of the barks are 70.55 and 132.23 µg/mL respectively for their antioxidant activity (Figure 7). The IC_{50} value for methanol extract of the leaves is closer than the same extract of barks to the IC_{50} value for the ascorbic acid. Thus, among the four extracts, methanol and hexane extract of AS leaves shows the highest and second highest antioxidant activities. The hexane extract of barks however, deviates strongly from ascorbic acid indicating their negligible antioxidant activity in comparison to the methanol extract of barks.

3.4 Anti-diabetic activity

The anti-diabetic activity for methanol extract of the leaves and barks are measured *in vitro* by taking acarbose (available anti-diabetic drug) as a standard drug. We used acarbose in α–amylase inhibition assay as it is a specific enzyme inhibitor to control post-prandial hyperglycemia and generally used by diabetic patients. The IC_{50} values are also calculated. The comparison between α–amylase inhibition activities of different extracts with acarbose is shown in Figure 8. From the study of anti-diabetic activity, IC_{50} value for the methanol extract of leaves and bark are found as 153.89 µg/mL and 123.91µg/mL respectively, as shown in Figure 9. The methanol extract of leaves shows comparatively very low anti-diabetic activity. Even though, these IC_{50} values for the methanol extract of leaves and bark are higher than the IC_{50} value of standard acarbose (50.22 µg/mL), they are significant enough to show considerable amount of enzyme inhibition activities (anti-diabetic activities). Alternatively, their role to remove the competitive inhibitor can help to restore the activity of the enzyme.

3.5 Antimicrobial assay

Pathogenic bacteria cause diseases to plants, animals and mostly human beings. Dysentery, tuberculosis, respiratory infections etc. are the most common diseases caused by pathogenic microorganisms. Antimicrobial agents present in the plant extracts inhibit or kill the growth of such microorganisms. The antimicrobial activity of the plant can be evaluated by calculating several parameters such as zone of inhibition (ZOI), minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The area around the antimicrobial disk where there is no growth of micro-organisms takes place is called ZOI, the minimum concentration of the plant extract that hinders the growth of microorganisms is called MIC and the minimum concentration of the plant extract that kills the microorganisms completely is called MBC. The ZOI, MIC and MBC values for the different bacterial species in methanol extracts of leaves and barks are tabulated (Table 2 and Table 3) below:

Table 2: Antimicrobial activity of methanol extract of leaves

S. N.	Bacteria	Reference Culture	ZOI value (mm)			MBC mg/mL	MIC mg/mL
			Positive Control Ampicilln	Negative Control Methanol	Plant extract		
1.	*Bacillus subtilis*	ATCC 6051	25	0	0	0	0
2.	*Escherichia coli*	ATCC 8739	27	0	18	0.1	10^{-5}
3.	*Klebsiella pneumonia*	ATCC 700603	18	0	13	1	10^{-4}
4.	*Salmonella typhii*	ATCC 29630	13	0	0	0	0
5.	*Staphylococcus aureus*	ATCC 6538P	23	0	22	0.01	10^{-4}

The ZOI of methanol extract of the leaves for two gram negative bacteria (*Escherichia coli, Klebsiella pneumonia*) and one gram positive bacteria (*Staphylococcus aureus*) are 18 mm, 13 mm and 23 mm respectively. Similarly, the same extract for these bacteria has MIC: 10^{-5} mg/mL, 10^{-4} mg/mL, 10^{-4} mg/mL and MBC: 0.1 mg/mL, 1 mg/mL, 0.01 mg/mL respectively. Moreover, the methanol extract of AS barks for these bacteria has ZOI: 16 mm, 12 mm and 24 mm; MIC: 10^{-4} mg/mL, 10^{-3} mg/mL and 10^{-6} mg/mL and MBC: 0.1 mg/mL, 1 mg/mL and 0.01 mg/mL respectively. These significant values of ZOI, MIC and MBC for the methanol extracts of leaves and barks confirm their antimicrobial activity i.e. the AS leaves and barks can be used to cure the diseases caused by *Escherichia coli, Klebsiella pneumonia* and *Staphylococcus aureus* bacteria. It is a strong evidence for supporting AS leaves and barks containing antibacterial agents.

Table 3: Antimicrobial activity of methanol extract of barks

S. N.	Bacteria	Reference Culture	ZOI value (mm)			MBC mg/mL	MIC mg/mL
			Positive Control Ampicillin	Negative Control Methanol	Plant extract		
1.	*Bacillus subtilis*	ATCC 6051	25	0	0	0	0
2.	*Escherichia coli*	ATCC 8739	27	0	16	0.1	10^{-4}
3.	*Klebsiella pneumona*	ATCC 700603	18	0	12	1	10^{-3}
4.	*Salmonela typhii*	ATCC 29630	13	0	0	0	0
5.	*Staphylococcus aureus*	ATCC 6538P	23	0	24	0.01	10^{-6}

3.6 Proximate nutritional analysis of the fruits

The proximate analysis is a set of methods to get information about the nutritional value of food. It uses some chemical-physical properties of a special group of nutrients. Even though, this analysis is not always selective: ammonia is also determined as protein and the nutritional value of the carbohydrate fraction is not determined precisely, we have followed it here as it is easy and cheap to conduct the analysis. The results obtained by the proximate analysis of the nutritional value of the *A. squamosa* fruits are summarized in Table 4. It can be ascertained that the AS fruits are very rich in nutritional value and their contents as low percentage fats and high percentage crude fiber stand them in the healthy fruits category. Additionally, comparatively less percentage of carbohydrate content makes such fruits very versatile with lot of health benefits especially for minimizing carbohydrates. Thus, they are ideal for people preferring low-sugar diets and if they are consumed in right amounts, it would be very valuable for the keto diet in which a person is required to eat less than 30 grams of carbohydrates per day.

Table 4: Percentage composition of nutritional parameters in the *A. squamosa* fruit

S.N.	Chemical Composition	Nutritional value/100g fruit
1.	Moisture	59.38
2.	Total ash	4.58
3.	Crude fat	0.22
4.	Protein	3.78
5.	Crude fiber	10.09
6.	Carbohydrate	21.95

As a rule, fruits in general are good sources for antioxidants, such as phenolic substances, vitamins, carotenoids, and minerals with potentially useful chemo preventive properties. In this work, we have not thoroughly studied antioxidant activities of the fruits but one of the major component of the antioxidants i.e. vitamin C is determined which is vital for our immune system, connective tissue, heart and blood vessels. The short procedure for determining vitamin C is explained below

D Determination of vitamin C

Weight of dry sample taken (W) = 10.0241 g

Volume of aliquot of ascorbic acid taken (V1) = 10 ml

Volume of Dye consumed by standard Ascorbic Acid (titre) (V2) = 4.8 ml

Volume of dye consumed by aliquot (V3) = 3.8 ml

Dye factor = 0.5/V2 = 0.5/4.8 = 0.1042

Amount of ascorbic acid per 100 g of fruits (mg)
= (Dye Factor×V3 ×V1)/W × 100
=(0.1042 ×3.8 ×10)/10.0241 × 100
= 39.50 mg.

Table 5: Amount of vitamin C content in different fruits

S.N.	Name of fruits	Amount of vitamin C (mg/100g fruit)
1.	*Annona squamosa* (Sugar apple)	39.5
2.	*Annona reticulata* (Custard apple)	19
3.	*Annona cherimola* (Cherimoya)	9
4.	*Ananus comosus* (Pineapple)	15
5.	*Persea americana* (Avocado)	8
6.	*Citrus paradisi* (Grapefruit)	34
7.	*Mangifera indica* (Mango)	28

The vitamin C content is estimated to be 39.5 mg of ascorbic acid per 100 g of *A. squamosa* fruits ('Ascorbic acid' is the technical term for vitamin C). According to the United States Department of Agriculture (USDA) nutrient database, any fruit which has from 6 to 15 mg of vitamin C is regarded as good source, 15 to 30 mg of vitamin C as a very good source and more than 30 mg as an excellent source of vitamin C [27]. Thus, the *A. squamosa* fruits can be considered as an excellent source of vitamin C. Some of the good, very good and excellent sources of vitamin C with its amount per 100 gram of the specific fruits are listed in Table 5 [25]. The *Annona squamosa* contains extremely higher amount of vitamin C in comparison to *Annona reticulata and Annona cherimola.* Hence, consuming *A. squamosa* fruit each day could be the best dietary supplement to fulfill the daily need of vitamin C for an adult (i.e. 60 mg/day to prevent clinical scurvy).

3.7 GC/MS Analysis

From the chromatograms and mass spectra for methanol extract of AS leaves (Figure 10 and Figure 11) and barks (Figure 12 and Figure 13), the identified major chemical compounds are listed in Table 6 and Table 7 respectively. The corresponding retention times and percentage area covered in the chromatogram are also listed.

Table 6: Compounds detected in GC/MS analysis of methanol extract of AS leaves

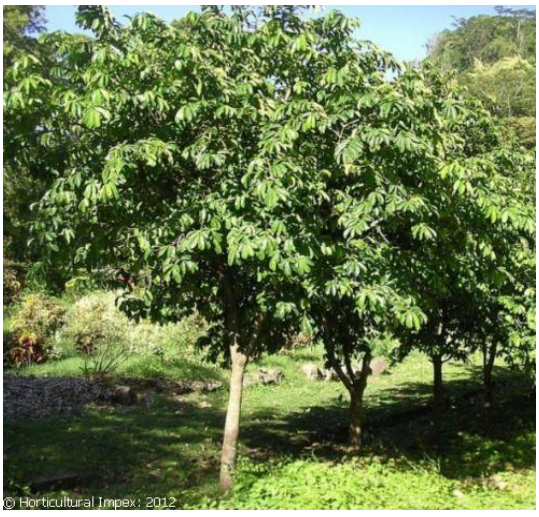
S.N.	Compounds	Retention Time	Percentage Area
1.	Germecra-4 (15), 5, 10, (14)-trien-1-α-ol	3.025	1.20
2.	Isospathunol <iso>	3.673	4.25
3.	6-Hydroxy-4, 4, 7a-trimethyl-5, 6, 7, 7a-tetrahydrobenzofuran-2 (4H)-one	3.810	1.99
4.	Methylhexadecanoate	4.455	2.49
5.	Palmitic acid	4.981	5.67
6.	Geranyl linalool <E, E>	5.603	8.33
7.	Methyllinoleate	6.256	4.44

8.	Linolenic acid (α)	6.359	3.32
9.	Phytol	6.440	13.17
10.	Caryophyllene (14-hydroxy-9 epi- Z)	7.234	0.55
11.	Napthalactone <4a-alpha-7-beta-7a-alpha>	7.405	0.97
12.	Elenodiol <8-alpha-11->	7.782	1.46
13.	Cedryl acetate	13.557	0.94
14.	Pogostol	21.645	0.95
15.	Nephytadiene	23.044	1.25

As shown in Table 6, the phytol with percentage area 13.17% appears as the most abundant chemical compound in the leaves. It is the product of chlorophyll metabolism and chemically called an acyclic diterpene alcohol that can be used as a precursor for the manufacture of synthetic forms of vitamin E [28] and vitamin K [29] which are very important in the many functions of the human body. It is essential in activating enzymes that have a positive effect on the production of insulin and is very effective in the decrease of blood cholesterol levels. Such tremendous application of phytol reflects that its natural resources such as AS leaves can be consumed directly as a medicine and used for developing specific drugs. Since, humans can convert free phytol into phytanic acid, patients with Refsum disease should limit their intake of free phytol [30], otherwise patients suffer from hearing loss, vision loss etc. due to the excessive accumulation of phytanic acid in cells and tissues. The phytol is also not recommended for very young children and those with immune system deficiency. Because of these side effects, all type patients and all age people should be very cautious while consuming AS leaves as a traditional medicine. Moreover, Geranyl linalool <E, E> with percentage area 8.33% is found as the second most abundant chemical compound in the leaves. It is a tertiary alcohol and diterpenoid in which one of the terminal methyl hydrogens is substituted by a geranyl group. It has a positive role as a metabolite and a fragrance and negative role as an amino acid inhibitor [31]. Similar analysis for the AS barks revealed that Isopimarol and Sandaracopimarinol exist as major chemical compounds with percentage area of 20.61% and 11.48% respectively, as summarized in Table 7. As the first compound "Isopimarol" has been reported as a strong antibacterial and antifungal agent [32] and the second compound "Sandaracopimarinol" has an inhibitory action to the growth of gram-positive bacteria but not responding to most of the gram-negative bacteria [33], the AS barks could be utilized to develop effective fungicides, disinfectants, antiseptics, or antibiotics. These explanations reflect that the AS plant extracts such as leaves and barks could be a potential prototype drug in pharmacology and pharmaceutics and can be used to derive the effective drugs and fragrances having different specific benefits.

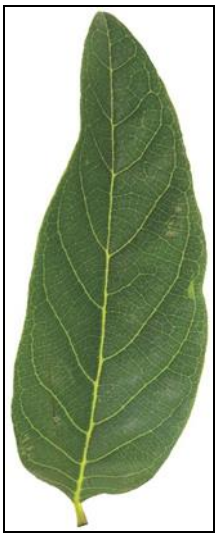
Table 7: Compounds detected in GC/MS analysis of methanol extract of AS bark

S.N.	Compounds	Retention Time	Percentage Area
1.	Germecra-4 (15), 5, 10, (14)-trien-1-α-ol	3.022	0.67
2.	Palmitate <methyl>	4.452	0.30
3.	Ximanyinic acid methyl ester	6.258	1.30
4.	Phytol	6.443	0.26
5.	Isopimarol	10.027	20.61
6.	Ledene oxide isomer	10.761	2.51
7.	Sandaracopimarinol	10.027	11.48
8.	Caryophyllene oxide	11.598	1.73
9.	Bisabolol acetate (α)	11.962	0.71
10.	Caryophyllene (14-hydroxy-9 epi- Z)	12.177	1.08
11.	Euderma-6, 11-diene <cis>	12.425	0.78
12.	Ionone <trans-beta>	13.900	2.16

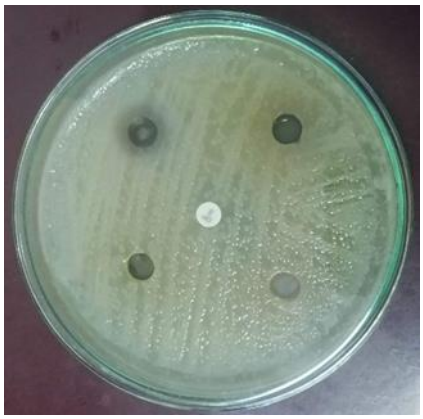


1a. A mature tree of *A. squamosa*

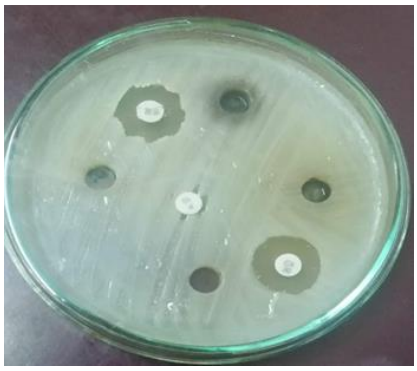
1b. A mature fruit and a flower of *A. squamosa*



1c. A mature leaf of *A. squamosa*



1d. Inoculation of plant extracts into the strain of *E. coli*



1e. Development of bacteria in the plant extracts

1f. MBC study of leaves extract on *E. coli*

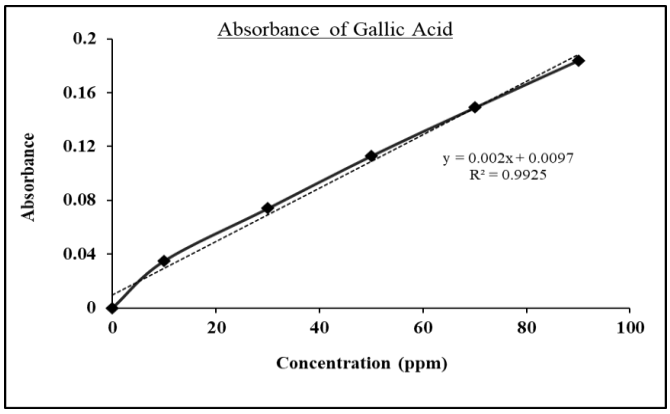


Fig 1: Calibration curve for Gallic acid

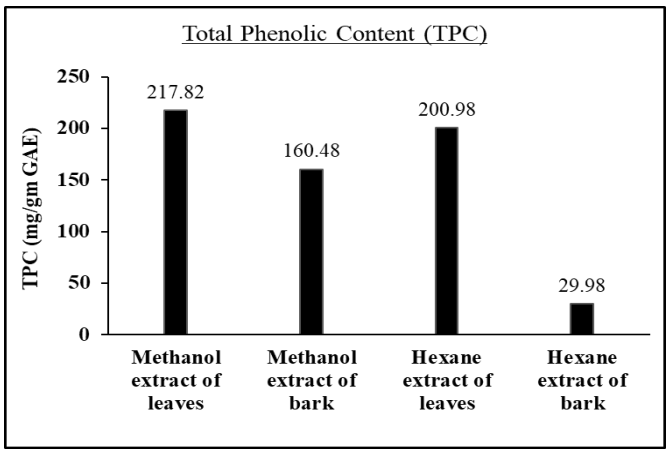


Fig 2: Total phenolic content (TPC) of different extracts of *A. squamosa*

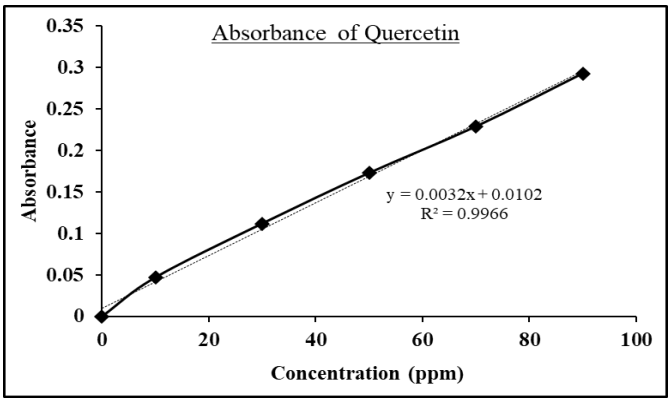


Fig 3: Calibration curve for Quercetin

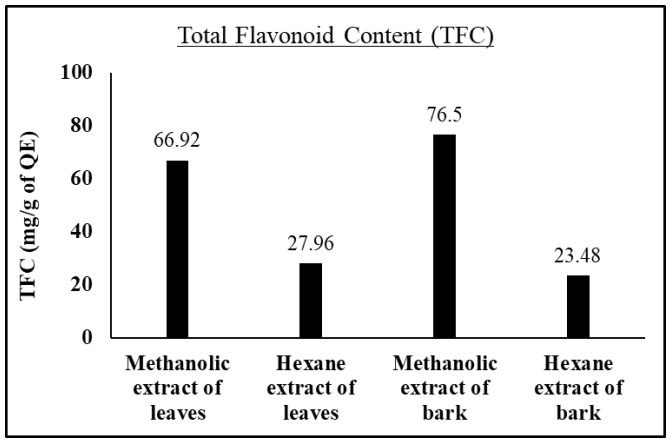


Fig 4: Total flavonoid content (TFC) of different extracts of *A. squamosa*

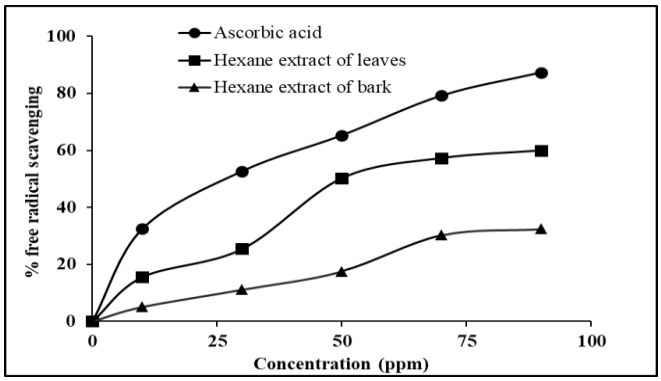


Fig 5: Comparison of antioxidant activity of hexane extracts with ascorbic acid

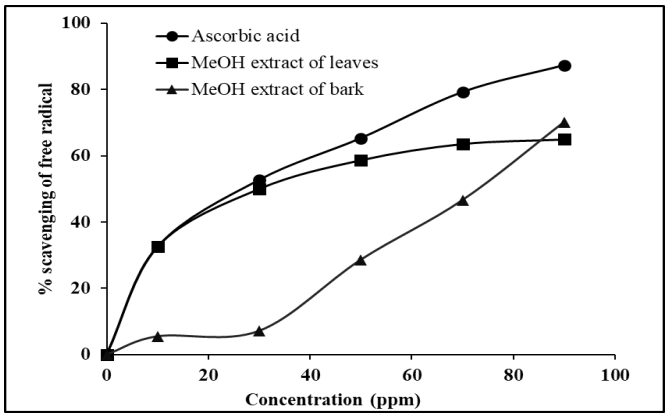


Fig 6: Comparison of antioxidant activity of methanol (MeOH) extracts with ascorbic acid

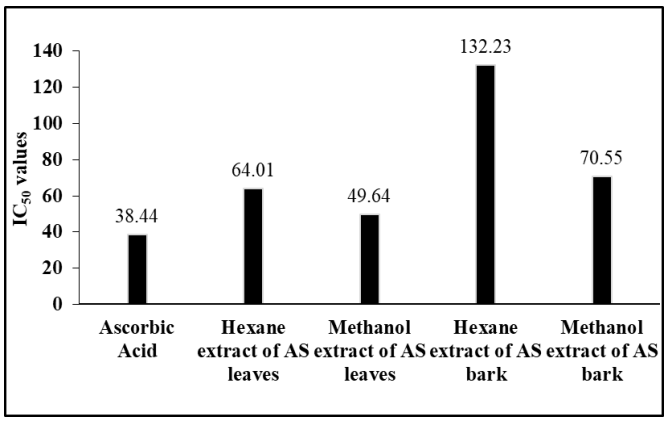


Fig 7: IC_{50} values for ascorbic acid, methanol and hexane extracts of *A. squamosa* leaves and bark

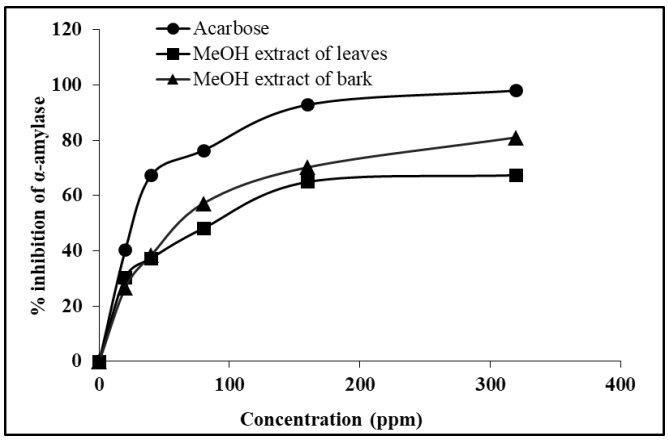


Fig 8: Comparison of Antioxidant activity of acarbose and methanol (MeOH) extracts of leaves and bark.

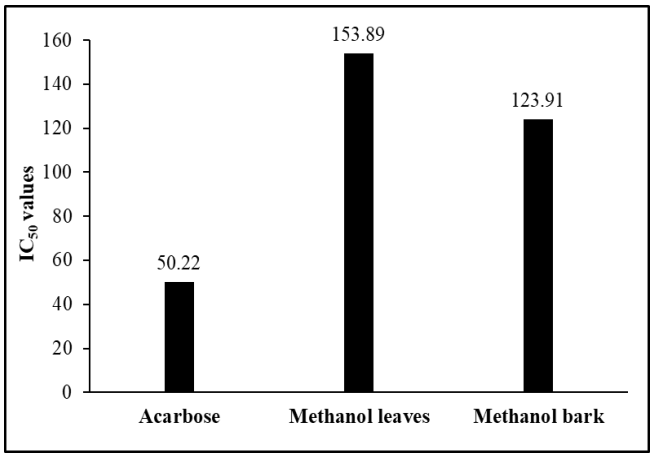


Fig 9: IC_{50} values for acarbose and methanol extracts of *A. squamosa* leaves and bark

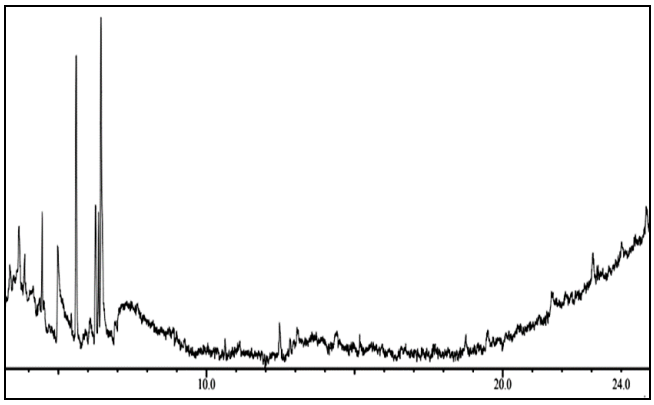


Fig 10: Gas Chromatogram for methanol extract of *A. squamosa* leaves

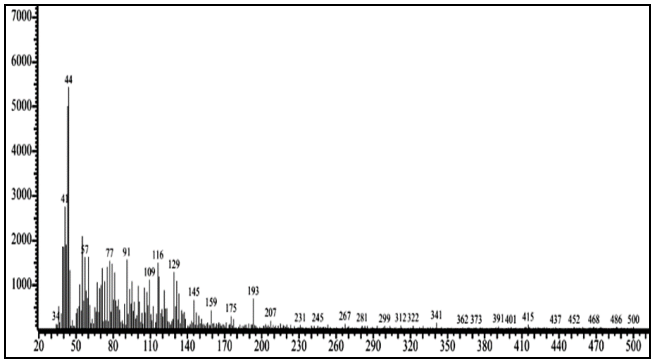


Fig 11: Mass spectrum for methanol extract of *A. squamosa* leaves

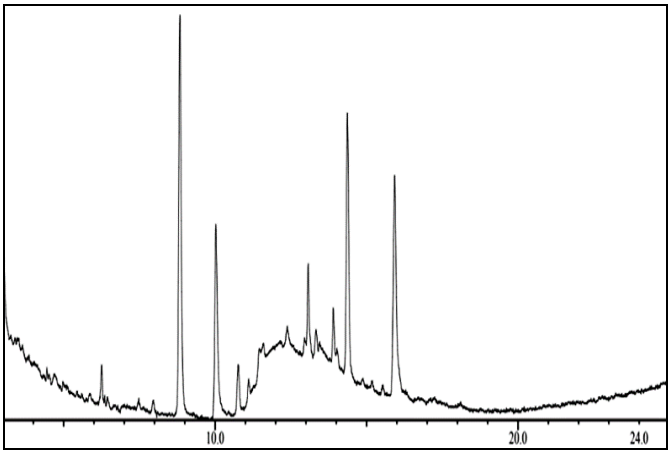


Fig 12: Gas Chromatogram for methanol extract of *A. squamosa* bark

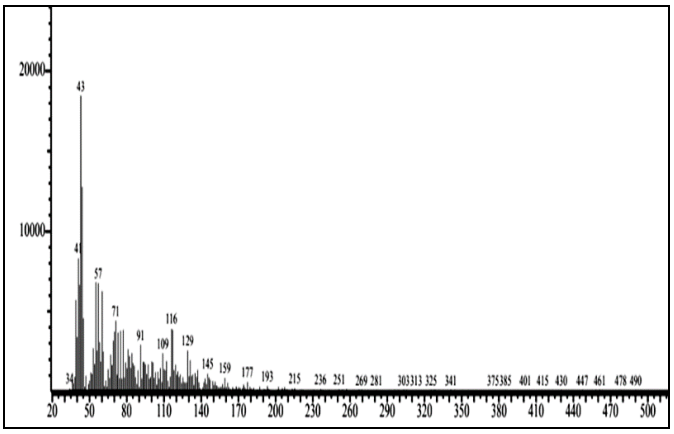


Fig 13: Mass spectrum for methanol extract of *A. squamosa* bark

4. Conclusion

The phytochemical screening for the methanol extract of leaves and bark of *Annona squamosa (SARIFA, सरीफा* in Nepalese language) has revealed carbohydrates, polyphenol, saponins, tannins, alkaloids, quinones, glycosides, phenolic compound and flavonoids and that for the hexane extract of leaves and bark has revealed polyphenol, glycosides, phenolic compounds and flavonoids as major phytochemicals. The total phenolic contents (TPC) for the methanol and hexane extract of leaves are found to be comparable whereas the TPC for methanol extract of leaves is higher than those of the bark. However, the total flavonoid contents (TFC) for the methanol extract of bark is higher than that of leaves. The IC_{50} values for the leaves and are found to be as comparable as their standards: ascorbic acid and acarbose, respectively. As the phenolic and flavonoid compounds are known to reduce the risk of oxidative stress induced diseases and for higher antidiabetic activity respectively, the methanol extracts of the leaves and bark could become the most potent natural antioxidant and antidiabetic agents respectively. It indicates that the *Annona squamosal* (AS) leaves could be used to develop different types of medicine that may prevent or delay some types of cell damage (antioxidant) and the AS bark could be used as a prototype drug for the treatment of diabetes (antidiabetic).

We evaluated the antimicrobial activity of the plant by calculating zone of inhibition (ZOI), minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The ZOI for methanol extract of leaves for two gram negative bacteria (*Escherichia coli, Klebsiella pneumonia*) and one gram positive bacteria (*Staphylococcus aureus*) are 18 mm, 13 mm and 23 mm respectively. Similarly, the same extract for these bacteria has MIC: 10^{-5} mg/mL, 10^{-4} mg/mL, 10^{-4} mg/mL and MBC: 0.1 mg/mL, 1 mg/mL, 0.01 mg/mL respectively. Moreover, the methanol extract of AS barks for these bacteria has ZOI: 16 mm, 12 mm and 24 mm; MIC: 10^{-4} mg/mL, 10^{-3} mg/mL and 10^{-6} mg/mL; and MBC: 0.1 mg/mL, 1 mg/mL and 0.01 mg/mL respectively. These significant values of ZOI, MIC and MBC for the methanol extracts of leaves and barks confirm their antimicrobial activity i.e. the AS leaves and bark can be used to cure the diseases caused by *Escherichia coli, Klebsiella pneumonia* and *Staphylococcus aureus* bacteria. The proximate nutritional analysis of the AS fruits predicted 4.58, 59.38, 10.09, 3.78, 21.95 and 0.22 percentage composition of different nutrition parameters: ash, moisture, crude fiber, crude protein, carbohydrate and fat respectively. This analysis ascertains that the AS fruits are very rich in nutritional value. The presence of low percentage fats and high percentage crude fiber stand AS fruits in the healthy fruits category. Additionally, presence of comparatively less percentage of carbohydrate content makes such fruits ideal for people preferring low-sugar diets. Therefore, these fruits can be recommended for the keto diet in which a person is required to eat less than 30 grams of carbohydrates per day.

From the GC/MS analysis of the AS extracts, phytol and geranyl linalool <E, E> are found to be the two most abundant chemical compounds in the leaves and is opimarol and Sandaracopimarinol are in the barks. The phytol being a precursor to manufacture synthetic forms of vitamin E and vitamin K and is essential in activating enzymes having a positive effect on the production of insulin and the geranyl linalool <E, E> having a positive role as a metabolite and a fragrance and a negative role as an amino acid inhibitor, their presence in the leaves makes AS best alternative medicinal plant. Furthermore, the isopimarol and sandaracopimarinol compounds found in the bark show strong antibacterial, antifungal activities and inhibitory action to the growth of gram-positive bacteria respectively, the AS barks could be a future potential prototype drug. These findings support the evidence of using AS leaves and barks as effective traditional medicines which ultimately form an important part of the world's healing process.

Even though it was desired to prepare the extracts of leaves and barks in other major solvents and extracts of roots, fruits peel and seeds to reveal wide varieties of phytochemicals; to assess antiallergic, antihelminthic, anticancer, antiseptic and anti-inflammatory tests and to study enzyme inhibition activities apart from α-amylase, we studied *in vitro* test only due to lack of enough research budget and time factor. The *in vivo* test can be carried out to further galvanize the results obtained here. Similarly, in nutritional analysis, to make proximate nutritional parameters more precise, qualitative and quantitative analysis of minerals and estimation of calorific value can be studied to assess other major vitamins.

5. Acknowledgement

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