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RESEARCH ARTICLE

Unveiling the Unexplored and Critically Endangered *Ilex khasiana* for its Antioxidant Properties

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Abstract

Ilex khasiana Purk. is an evergreen tree of Aquifoliaceae family reportedly endemic to Khasi Hills, Meghalaya, India; but it is also seen in a localised area of Aizawl, Mizoram, India. There is a vast history about the genus *Ilex* with reference to their medicinal uses in different traditional practices such as, in the treatment of coronary heart diseases, hypertension, hyperlipemia and hepatitis. Interestingly, *I. khasiana* is relatively unknown and yet it is known to possess multiple medicinal characteristics. The antioxidant potential studies were conducted on leaf extract specimens from Mizoram, using petroleum ether (IKP), chloroform (IKC) and methanol (IKM). IKM had the highest total antioxidant activity with 76.42 ascorbic acid equivalent (AE) mg/g, followed by IKP with 44.27 AE mg/g, and then by IKC with 17.08 AE mg/g respectively. The reducing power assay showed a concentration-dependent activity against potassium ferricyanide. The total phenolic content was found to be 3.46 gallic acid equivalent (GAE) mg/g for IKM and 1.450 GAE mg/g for IKC respectively. The total flavonoid content was 41.9 quercetin equivalent (QE) mg/g for IKC and 30.8 QE mg/g for IKM. IKP did not show any activity in phenolic and total flavonoid assays.

Keywords: Antioxidant Activity, Flavonoids, *Ilex khasiana,* Phenolics, Reducing Power

1. Introduction

The nature and activity of bioactive compounds present in plants play a very important role not only in curing but also in preventing the occurrence of many diseases. Apart from their diverse biological activities, these bioactive compounds share a common medicinal property in possessing antioxidant activities, which is the ability of scavenging deleterious free radicals produced in all metabolising cells. These free radicals or oxidants have a propensity to react with most biological compounds by donating or accepting electrons and these include fundamental biomolecules such as lipids, proteins, and nucleic acids[1]. They are therefore a major factor in the etiology of numerous chronic diseases including arthritis, autoimmune disorders like multiple sclerosis and rheumatoid arthritis, cardiovascular diseases, diabetes mellitus,

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neurological diseases, respiratory diseases, cataract development, and cancers. Antioxidants from plants not only impede these radicals from their ill-effects but also slows down the apoptotic process, thereby preventing further detriments[2]. Thus, validating the antioxidant potency of medicinal plants is crucial for the development of better or even new drugs[3].

Species of *Ilex* are well known in traditional Chinese medicine and have served as botanical sources of several compounds of health benefits[4]. Among the 410 nominal species under the genus *Ilex* indexed in The Plant List[5]. *I. khasiana* Purk. (family Aquifoliaceae) is recorded as a holy species endemic to the Khasi Hills of Meghalaya, India, and classified as critically endangered under the IUCN Red List of Threatened Species[6]. *I. khasiana* is an evergreen tree with an average height of 15–20 m and forming the sub-canopy in the humid subtropical forests at an elevation up to 1990 m above the sea level[7]. The tree starts flowering in summer during April-May, and fruits develop in winter during November-December. The fruits are purplish red with a size of 7–8 mm and the seeds, which are 3 mm long, are obovoid-ellipsoid or ellipsoid[8]. The aerial plant parts (mainly the fruit) serves as fodder for wild animals like palm civets, squirrels, and birds. Among the Khasi people, the bark and root decoction are used in the treatment of tuberculosis and severe cold[9]. The species has also been identified from a localised area in Aizawl, Mizoram. Among the Mizo traditional healers, it is known as a good medicine but the exact ailment to which the plant is used remains unknown[10].

In fact, other species under the genus *Ilex* show quite a wide range of medicinal values. *I. pubescens* is used for the treatment of coronary heart diseases, and as anti-inflammatory and analgesic agent in traditional Chinese medicine[11,12]. Its leaves contain phenolics and phenylpropanoids which are responsible for its antipyretic, anti-inflammatory, analgesic, cardiovascular and circulatory activities. The four substituted catechols in the plant may be responsible for the vasodilator and hyperanemic effects[13]. It is also known to be effective in the treatment of hypertension, hyperlipemia, and hepatitis[14]. *I. cornuta* is been used in Chinese Traditional medicine for the treatment for dizziness and hypertension[15]. *I. ficoidea* and *I. centrochinensis* has shown potent anti-inflammatory and antioxidant activities[16]. *I. paraguariensis* has shown anti-obesity effects in mice[17]. In the light of these information, it is worthwhile to investigate *I. khasiana.*

2. Materials and Methods

2.1 Collection of Plants

Ilex khasiana is available as a naturally propagated plant only in Mizoram, India, at Luangmual, Aizawl (23°44.556'N and 92°41.956'E). The plant was collected and authenticated at the Botanical Survey of India, Eastern Circle, Shillong, Meghalaya. The herbarium catalogue is maintained at the Department of Pharmacy, Regional Institute of Paramedical and Nursing Sciences, Zemabawk, Mizoram, India, with reference No. BSI/EC/Tech./2008/577.

2.2 Preparation of Plant Extract

The leaves were washed, dried under shade and ground to a coarse powder. Soxhlet extraction was employed using three solvents - petroleum ether, chloroform, and methanol for 72 hours each. The solvents were removed, and the extracts were concentrated in a rotary vacuum evaporator (Buchi Rotavapor® R-215). The three extracts *I. khasiana* petroleum ether extract (IKP), *I. khasiana* Chloroform Extract (IKC) and *I. khasiana* Methanol Extract (IKM) yielded semi-solid extracts and these were stored at 4 °C until further use.

2.3 Determination of Total Antioxidant Activity

Phosphomolybdate estimation was carried out by the method of Prieto *et al.* with slight modification[18]. Standard ascorbic acid was prepared in a series of concentration, *viz.* 10, 20, 40, 60, 80 and 100 µg/mL from which 0.1 mL was taken and mixed with a reagent composed of 0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate. The mixture was incubated at 95°C for 90 minutes. The absorbance was measured using UV-vis spectrophotometer (Labtronics LT-2700) at 695 nm to plot a standard curve. The same method was followed by taking 100 µL of IKP, IKM, and IKC from a stock solution of 1 mg/mL respectively. Total antioxidant

activity was presented as milligrams of ascorbic acid equivalent to per gram of the dried extract.

2.4 Determination of Reducing Power

The reducing power was estimated following the modified methods of Oyaizu using ascorbic acid as standard[19]. 1 mL each of IKP, IKM, IKC and ascorbic acid of varying concentrations such as 10, 20, 40, 60, 80 and 100 µg/mL was taken. To this 2.5 mL of phosphate buffer (6.6 pH) and 2.5 mL of 1% potassium ferricyanide were added. After 30 minutes of incubation at 50 °C, 2.5 mL of 10% tri-chloroacetic acid was added to terminate the reaction. The samples were centrifuged at 3000 rpm for 10 minutes. 2.5 mL of the supernatant was taken and mixed with 2.5 mL of distilled water after which it was vortexed with 0.5 mL of freshly prepared 0.1% ferric chloride solution. The absorbance was measured at 700 nm[19]. Higher absorbance indicated the increase in reducing power.

2.5 Determination of Total Phenolic Content

Total phenolic content of the plant extract was determined using gallic acid as standard following Folin-Ciocalteu assay according to the modified method of Singleton *et al*[20]. 1 mL of the plant extracts (50 µg/mL) and gallic acid with concentrations of 10, 20, 30, 40, 60, 80 and 100 µg/mL was taken. A tenfold diluted 5 mL Folin-Ciocalteu reagent was added to all. After three minutes, 4 mL of 0.7 M sodium carbonate solution was added to the mixture and incubated for 1 hour at room temperature. The absorbance was measured at 756 nm. Absorbance of gallic acid at different concentrations was used to plot a standard curve from which the total phenolic content of the plant extracts was calculated and expressed as milligrams of gallic acid equivalent (GAE) per g of the dried extract.

2.6 Determination of Total Flavonoid Content

Quercetin was used as a standard to estimate the total flavonoid content of *Ilex khasiana* extract using aluminium chloride ($AlCl_3$) assay based on the modified method of Zhishen *et al*.[21] To 1 mL of the plant extract (50 µg/mL), 2 mL of distilled water was added. After 5 minutes at room temperature, 3 ml of 5% sodium nitrite ($NaNO_2$) and 0.3 mL of 10% $AlCl_3$ were added to the mixture. 6 minutes later 2 mL of NaOH (1 M) was added and the volume was made up to 10 mL with distilled water. 1 hour after the incubation, absorbance was measured at 510 nm. The same procedure was employed to the standard in a series of concentration (5, 10, 20, 40, 60, 80, and 100 µg/mL). The absorbance of different concentrations of quercetin provided the standard curve and the total flavonoid content was expressed as milligrams of quercetin equivalent (QE) per gram of the dried extract.

3. Results

3.1 Total Antioxidant Activity

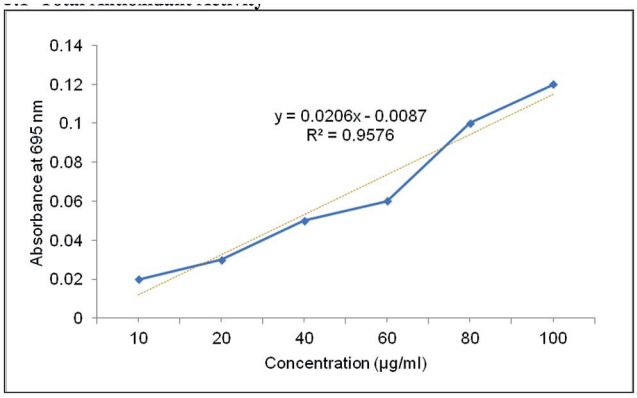

Figure 1. Standard curve of ascorbic acid for total antioxidant assay. Dotted line represents the linear graph. Values are in mean ± standard deviation; $n = 3$.

The total antioxidant activity of the *I. khasiana* was estimated from three different solvent extracts. The value was presented as milligrams of ascorbic acid equivalent (AE) per gram of the dried extract (Figure 1). IKP showed 44.27 AE mg/g, IKC showed 17.08 AE mg/g, and IKM showed 76.42 AE mg/g of antioxidant activity respectively.

3.2 Reducing Power

The plant extract showed positive reducing power against potassium ferricyanide which increases with concentration. However, ascorbic acid indicated high activity, while *I. khasiana* extract showed only minimal activity (Figure 2).

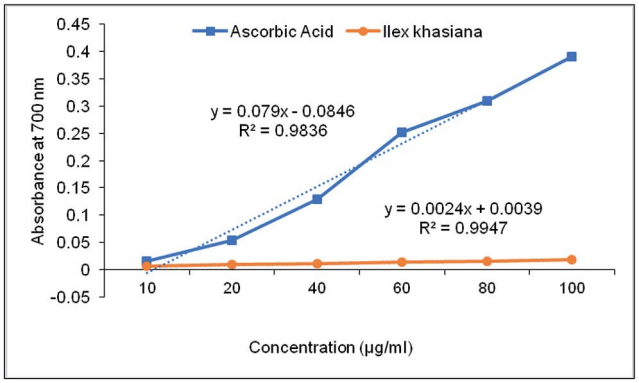

Figure 2. Potassium ferri-cyanide-reducing activity of ascorbic acid and I. khasiana extract. Line with square markings indicates the activity of ascorbic acid while line with circle markers indicate that of I. khasiana. Values are in mean ± standard deviation; n = 3.

3.3 Total Phenolic Content

The total phenolic content of the plant extract was calculated from the standard curve of gallic acid (Figure 3) and expressed as milligrams of gallic acid equivalent (GAE)/g of the dried extract. It was found that IKM contained 3.46 GAE mg/g and IKC contained 1.450 GAE mg/g. However, IKP did not show any activity.

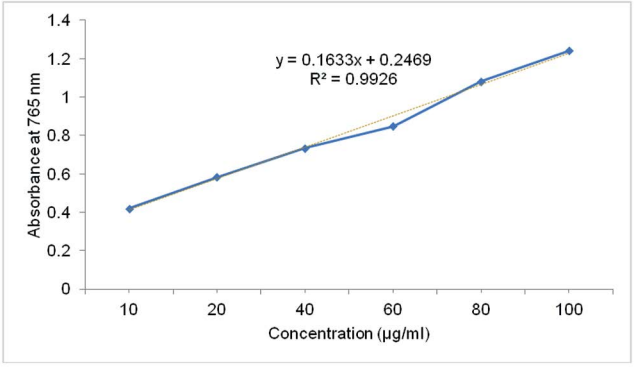

Figure 3. Standard curve of gallic acid for total phenol assay. Dotted line represents the linear graph. Values are in mean ± standard deviation; *n* = 3.

3.4 Total Flavonoid Content

The total flavonoid content of the plant extract was estimated against quercetin as a standard antioxidant (Figure 4), and the value is expressed as milligrams of QE per gram of the dried extract. From the standard curve, it was calculated that IKM had 30.8 QE mg/g and IKC had 41.9 QE mg/g. IKP again did not show any activity.

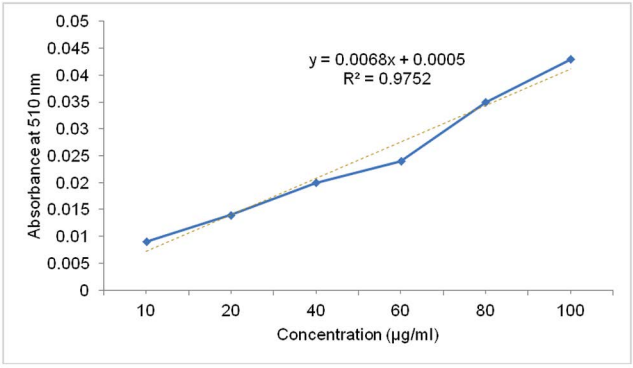

Figure 4. Standard curve of quercetin for total flavonoid assay. Dotted line represents the linear graph. Values are in mean ± standard deviation; n = 3.

4. Discussion

Among the genus of *Ilex*, *I. khasiana* is virtually an unexplored species in comparison with the other well-known species which all exhibit remarkable medicinal and nutritive values. It is a critically endangered plant which is endemic to Meghalaya, and is also found in Aizawl, Mizoram. There are no records of an in-depth study of this plant with regards to its medicinal value. As an endangered species the development of protocols for *in vitro* mass propagation of the species to augment the species population has been described[22]. The medicinal potentiality of this plant may increase when it is taken into account the wide range of medicinal properties of other related species of *Ilex* like *I. pubescens, I. aquifolium, I. integra,* and *I. paraguariensis*. These plants are known for their usefulness in the treatment of coronary heart diseases, hypertension, hyperlipemia and hepatitis. Phenolics and phenylpropanoids present in the plants account for antipyretic, anti-inflammatory, analgesic, cardiovascular and circulatory activities[11,12].

Many pathological issues that we encounter in life today may be attributed to free radicals which are involved in aging process[22]. Free radicals react with almost every cell component thereby contributing to various diseases depending upon their target. If the target is DNA, the probability of cancer increases, and if it is low-density lipoprotein (LDL) in blood then arteriosclerosis may result. Meanwhile, a decrease

in blood antioxidant may worsen hyperglycaemic condition in diabetes[23]. Fortunately, the high antioxidant activity of *I. khasiana* extract revealed in this study may aid in relieving the unending quest for the treatment of the medical conditions due to free radicals.

Moreover, the relationship between antioxidant and pro-oxidant in a biological system is a never-ending combat in which antioxidants can reduce the pro-oxidants but not Fe^{3+} effectively[24,25]. This is because not all reductants are antioxidants, but all antioxidants are reductants. Therefore, bioactive compounds or food components play a crucial role in maintaining the redox-equilibrium in the endobiotic system as they possess the electron-donating capability associated with their antioxidant property[26].

The mode of action of phenolics as antioxidants can be in multiple ways. In one way, they donate hydrogen from their hydroxyl group which reacts with reactive oxygen and reactive nitrogen species breaking the cycle of new radical generation[27–29]. On the other hand, the metal-chelating ability of phenol reduces the production of free radicals and the hydrophobic benzenoid rings. Hydrogen-bonding potential of the phenolic hydroxyl groups has a strong potency to interact with proteins and acts as an antioxidant by inhibiting enzymes such as lipoxygenases, and cyclooxygenase[30,31]. Therefore, the phenolics in *I. khasiana* are most probably responsible for many of the antioxidant activities found in this study.

Anti-inflammatory, anti-oestrogenic, enzyme inhibition, antimicrobial, anti-allergic, vascular and cytotoxic antitumor activity are the most prominent attributes of flavonoids besides their UV screening properties and their contribution to attracting pollinators[32,33]. Besides their activity on the prevention of oxidative stress, flavonoids act as anticancer agents through different pathways like repressing tumor and oncogenes formation, redox reaction, regulating hormone metabolism, apoptotic induction, and stimulation of DNA repair and boosting immune system[34]. The high flavonoid content of *I. khasiana* therefore indicates it is important medicinal values for the treatment of complex diseases.

5. Conclusion

Our study shows that *I. khasiana* leaves contains bioactive compounds that are capable of scavenging free radicals. An inherent nuisance to the proper functions of cells, free radicals have to be effectively eliminated from the body. Debilitating diseases involving immunity, central nervous system, and cancer are primed by unchecked free radicals. The antioxidant activities observed in this study indicate the plant extract as a potential source of lead molecules for important health benefits. In addition, the species is critically endangered and found only in Meghalaya and Mizoram, which renders it more interesting to study about its pharmacology as well as its conservation and propagation.

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