Ilex khasiana - The silent holly species of Aquifoliaceae on its pharmacognostical importance as a free radical scavenger and antibacterial agent

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**ABSTRACT**

*Ilex khasiana* is a rare and endangered medicinal plant endemic to northeast India. Remaining largely unexplored, it is purported to have a range of medicinal values and may soon join the medicinal plant hall of fame. Extracts of the leaves were prepared using solvents of increasing polarity, namely petroleum ether (IKP), chloroform (IKC) and methanol (IKM). The preliminary phytochemical screening indicated the presence of alkaloids, phytosterols, triterpenoids, saponins, reducing sugars, glycosides and carbohydrates. Free radical-scavenging activity was assessed using 2,2-diphenyl-1-picrylhydrazyl (DPPH). IKC exhibited the highest activity with an IC_{50} of 17.22 ± 1.87, followed by IKM with an IC_{50} of 26.93 ± 5.14 and IKP with an IC_{50} of 37.16 ± 5.11. Butylated hydroxytoluene (BHT) was used as the standard DPPH scavenger and showed an IC_{50} of 8.31 ± 0.72. IKM and IKC also showed positive antibacterial activity against Gram-negative bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella*, and a Gram-positive bacteria *Bacillus subtilis*. IKP did not indicate any inhibition against the selected bacteria. Our findings substantiate the basis for further investigations on the medicinal potentials of the plant.

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**INTRODUCTION**

Natural products from medicinal plants have become a front-liner in our quest to obtain a reliable source of remedies. With the ever-evolving pathogens as well as human lifestyles, it is an urgent outcry to seek the aid of natural products to tackle the eternal battle to have a better, healthier living. Advancement in the knowledge of free radicals and reactive oxygen species (ROS) concerning disease management marked a milestone in pharmaceutical development (Aruoma, 2003). When the equilibrium between free radicals and antioxidants is disturbed in a physiological system, a condition called oxidative stress occurs, which causes an alteration in proteins, DNA, and lipids. These changes become the basis of various human diseases (Lobo et al., 2010). Even though there is a biological barrier to combat these free radicals using proteins like catalases, superoxide dismutase, and glutathione peroxidase, the human body requires an alternative source of antioxidants to effectively neutralize the...
free radicals which are readily available in the form of secondary metabolites like flavonoids and terpenoids.

In addition, the involvement of microbes in human health is still an undeniable factor contributing to a high number of health problems and death cases in the world every year (WHO, 2004). The emergence of multidrug resistance (MDR) leaves human health at a great peril which will greatly affect an individual as well as the economy (Anand et al., 2019). A recent study shows that by 2050, 100 trillion dollars will be spent on account of antimicrobial resistance (AMR) which will be responsible for the death of 10 million lives per year (de Kraker et al., 2016). Considering the present scenario, it will be a wise move to employ bioactive compounds to stand against the fast-emerging multidrug resistance which already contributes 50% of the drug approved by Food and Drug Administration (Chavan et al., 2018).

This is where Ilex khasiana Purk (Aquifoliaceae) comes in, having a long lineage of remarkable contribution to human health in the form of natural remedies. Its closely related Ilex species such as I. pubescens, I. cornuta, I. ficoidea, I. centrochinensis, and I. paraguariensis are known to have antipyretic, anti-inflammatory, analgesic, anti-obesity, cardiovascular, and circulatory activities (Koo et al., 1985; Li et al., 2011; Bracesco et al., 2011). Also, I. paraguariensis and I. vomitoria are known to possess anti-inflammatory, antioxidant, and anticancer activities in which chlorogenic acid, quercetin, kaempferol, and their glycosides may play a vital role (Bravo et al., 2007; Yi et al., 2016; Noratto et al., 2011).

Few studies are done on the antioxidant and antimicrobial activities in which Ilex species are known to have a significant effect. I. paraguariensis is known to possess antimicrobial activity against selected food pathogens such as Staphylococcus aureus, Listeria monocytogenes, Salmonella enteritidis, and Escherichia coli (Burris et al., 2011). The ethanol, ethyl acetate, chloroform, and n-hexane extracts of I. aquifolium were found to be effective against E. coli, S. aureus, Enterobacter aerogenes, Proteus vulgaris, Salmonella typhimurium, and Candida albicans (Erdemoglu et al., 2009). A recent study showed that I. khasiana has remarkable antioxidant properties which makes it suitable to compete with other species (Lalnunfela et al., 2019). Therefore, further investigations on these medicinal properties are necessary.

MATERIALS AND METHODS

Plant material and extraction

The only known full-grown Ilex khasiana is found at Luangmal, Aizawl, Mizoram, India (location 23°44.556’N and 92°41.956’E). The plant specimen was authenticated at the Botanical Survey of India, Eastern Circle, Shillong, Meghalaya, with reference No. BSI/EC/Tech./2008/577.

![Figure 1: Percentage inhibition DPPH by I. khasiana extracts (IKM, IKC, IKP) and BHT. Values are expressed as means ± SEM (n=3).](image1)

![Figure 2: Comparison of IC50 of I. khasiana extracts (IKM, IKC, IKP) on their free radical-scavenging activity against DPPH. Values are expressed as means ± SEM (n=3).](image2)

The leaves were collected and dried in the shade. The dried leaves were made into a coarse powder. Plant extraction was done in a Soxhlet apparatus for 72 hours. Solvents of varying polarities were used, namely petroleum ether (highly non-polar), chloroform (polar), and methanol (non-polar). The extracts were concentrated by removing and recovering the solvents in a rotary vacuum evaporator (Buchi Rotavapor® R-215). The three semi-solid extracts namely I. khasiana petroleum ether extract (IKP), I. khasiana chloroform extract (IKC), and I. khasiana methanol extract (IKM) so obtained were stored at 4°C until further use.
Phytochemical Screening

Standard protocols were followed for screening the presence of phytochemical compounds. Meyer’s test and Dragendorff’s test were used for alkaloids; Liebermann-Burchard’s test for phytosterols; Shinoda test for flavonoids; screening for gums; Benedict’s test, zinc hydrochloride reduction test, and Fehling’s test for reducing sugar; tannins by FeCl₃ and K₂Cr₃O₇ test; foam test for saponin; Liebermann test for triterpenoids; Biuret test and ninhydrin test for amino acids; Legal’s test and Keller-Killiani’s test for glycosides; Wagner’s test, Hager’s test, Molisch’s test, Fehling’s test, and Barfoed’s test for carbohydrates.

Free radical-scavenging activity

Estimation of the free radical-scavenging activity was performed using the method of Blois with slight modification (Blois, 1958). DPPH (2,2-diphenyl-1-picrylhydrazyl) was the free radical used as a substrate, and butylated hydroxytoluene (BHT) was used as a reference standard. The plant extracts were prepared in different concentrations (viz. 10, 20, 30, 40, 50, 80, and 100 µg/ml). One ml of 0.1 mM DPPH in methanol was mixed with 3 ml of the plant extracts. They were left to stand still at room temperature for 30 minutes. A blank solution was prepared in the same manner without the extract. The mean value of absorbance was obtained by measuring at 517 nm against control in a UV-visible spectrophotometer (Evolution™, Thermo Scientific). The percentage of inhibition (I) was calculated by the equation,

\[ I = \left( \frac{Abs \ control - Abs \ sample}{Abs \ control} \right) \times 100 \]

Antibacterial activity

The antibacterial potency of the different extracts of *Ilex khasiana* was determined using the disk diffusion method. Four bacteria, namely *Escherichia coli* (ATCC-25922), *Pseudomonas aeruginosa* (ATCC-15442), *Klebsiella pneumoniae* (ATCC-BAA-1705), and *Bacillus subtilis* (ATCC-6051) were subjected to the test. The bacteria were cultured in sterile Petri culture plates containing Mueller-Hinton agar. Two concentrations, i.e. 10 and 20 mg/ml, were prepared for each extract. The extracts were inoculated on sterile disks and were placed on the culture disk along with standard ceftriaxone (10 µg) disks. The plates were incubated at 37 ± 1°C for 20 hours, and the corresponding zones of inhibitions were measured.

Statistical analysis

Data were recorded in means ± standard error of the means (SEM). Results were subjected to a one-
way analysis of variance, and the mean comparisons were performed by Tukey’s multiple range test using SPSS version 19.0 (Statistical Package for the Social Sciences, Inc., Chicago, Illinois, United States). Differences between means were considered significant at \( p \) value less than 0.05.

**RESULTS**

The preliminary phytochemical screening of *Ilex khasiana* leaves showed the presence of pharmaceutically important phytochemical compounds including alkaloids, phytosterols, reducing sugar, saponins, triterpenoids, glycosides, and carbohydrates. Flavonoids, amino acids, gums, and tannins were not detected from the standard tests applied. Among the three extracts, the IKM was found to possess the maximum number of bioactive compounds (Table 1). This shows that solvent polarity, as methanol was the most polar, has a major influence in the extraction of the chemical constituents.

**Free radical-scavenging activity**

A free radical-scavenging assay based on DPPH scavenging was used to test the activity of *I. khasiana*. The three extracts prepared in concentrations of 10, 20, 40, 60, 80, and 100 \( \mu \)g/ml showed concentration-dependent activity as the scavenging activity increased with an increase in concentration (Figure 1). \( IC_{50} \) was calculated for all the extracts as well as the standard drug. BHT showed the highest activity with the lowest \( IC_{50} \) of 8.31 \( \pm \) 0.72. Among the plant extracts, IKC was found to have the best scavenging activity, having an \( IC_{50} \) of 17.22 \( \pm \) 1.87, followed by IKM with an \( IC_{50} \) of 26.93 \( \pm \) 5.14 and lastly IKP having the highest \( IC_{50} \) of 37.16 \( \pm \) 1.3848 (Figure 2).

**Antibacterial activity**

The antibacterial activity of *I. khasiana* extracts was performed on four selected species, namely *B. subtilis*, *P. aeruginosa*, *E. coli*, and *K. pneumoniae*. Both IKM and IKC showed concentration-dependent activity (Figures 3 and 4). IKC showed better activity on *B. subtilis* and *E. coli* (with inhibition zones of 5.95 \( \pm \) 1.88 mm and 7.38 \( \pm \) 0.26 mm at 10 and 20 mg/ml respectively), while IKM had higher activity against *K. pneumoniae* and *P. aeruginosa* (8.99 \( \pm \) 0.79 mm and 6.88 \( \pm \) 0.37 mm respectively) (Figure 5). IKP showed no inhibition on the proliferation of any of the bacteria. The specific antibacterial property of *I. khasiana* thus depends upon the type of solvent used for extraction, implying that the antibacterial principle(s) must be a polar compound.

**DISCUSSION**

The medicinal properties of plants will play a major role in the days to come as there is a greater demand for products with minimum side effects and higher potency to overcome the disadvantages found in modern synthetic drugs (Appendino et al., 2014). The prospects of medicinal plants for the development of novel drugs are promising. Thus, in-depth research is going to surface the antioxidant and antimicrobial activity of plants that are available in nature abundantly.

Molecules that are produced from the metabolism of oxygen called reactive oxygen species (ROS) are highly reactive and influence a vast role in human health. ROS are known to damage cell and tissue, involving in many degenerative ailments like Alzheimer’s disease, heart problem, cellular mutations and cancer (Harman, 1994; Cox and Cohen, 1996; Ames, 1998). Even though there are several physiological armories, enzymatic such as superoxide dismutase, catalase, and glutathione peroxidase and nonenzymatic such as ascorbic acid and glutathione, that fight against ROS activity (Cesaratto et al., 2004), they are not sufficient to ward off the bulk of endogenous and additional exogenous ROS. Thus, there is an increasing demand for antioxidants from medicinal plants for a better life.

Plant secondary metabolites as antimicrobial agents is another breakthrough in pharmaceutical sciences where the present antibiotics failed to perform their duty due to multidrug resistance (Appendino et al., 2014). Plants extracts, coupled with hackneyed antibiotics, are found to escalate the antimicrobial activity (Dudhatra et al., 2012; Tatiraju et al., 2013). These metabolites are not required by plants for proper functioning and development, but protection from harmful pathogens like bacteria and virus and help in reproduction (Boy et al., 2018; Mawalagedera et al., 2019). Thus, screening of plants for their antioxidant and antimicrobial activities is the primary and pivotal step to achieve pharmaceutical breakthroughs (Nenaah and Ahmed, 2011).

The microbicidal effect of a plant depends highly on the solubility of the bioactive compounds (House, 2002), which is the reason why *I. khasiana* extracts IKM, IKC, and IKP have a different impact on the selected microbes. The extracted compounds, depending upon their morphology and size, will determine the penetration potential on the surface of the microbe (Kavak et al., 2010). In many cases, plant extracts become less effective because of the hydrophilic cell wall of Gram-negative bacteria (Sumbul et al., 2011). Plants like cinnamon, lavender, oregano, and rosemary are reported to
have a lower effect on Gram-negative bacteria than Gram-positive bacteria (Burt et al., 2007). But the present study indicates that *I. khasiana* has a bactericidal effect on all three selected Gram-negative bacteria, namely *P. aeruginosa*, *E. coli*, and *K. pneumoniae* and also on a Gram-positive bacteria *B. subtilis*. Other species of *Ilex* have been reported to have antibacterial activity. Among them, *I. paraguariensis* a famous beverage that is preferred over tea and coffee and it is known to have an antimicrobial effect on both Gram-positive and Gram-negative bacteria (Biasi et al., 2009; Cogo et al., 2010). The phenolic compound present in this plant is represented by ferulic acid, gallic acid, syringic acid, and ρ-coumaric acid (Pagliosa et al., 2010). *I. aquifolium* is also known to have triterpenoids derivatives like oleanolic acid and ursolic acid, which are accountable for its antibacterial and antifungal activities (Knights and Smith, 1977). Moreover, *I. brasiliensis* and *I. paraguariensis* are found to have a protective impact against ischemia-reperfusion on the myocardium and other oxidative damages attenuation which may be the result of antioxidant activities of the extract (Schinella et al., 2009). Caffeoylquinic acid derivatives in *I. guayusa* are known to have antioxidant, anti-inflammatory and anti-diabetic properties. Chlorogenic acid, in particular, has been reported to accelerate insulin sensitivity and glucose tolerance while inhibiting gluconeogenesis in diabetic mice (Ong et al., 2013; Domingueti et al., 2016).

### CONCLUSIONS

We found in this study that *Ilex khasiana* has a high value as a medicinal plant as indicated by its free radical-scavenging activity and broad-spectrum antibacterial activity. The antibacterial property is notable as it is effective against both Gram-positive and Gram-negative bacteria. Considering our research findings, the obscure *I. khasiana* is clearly a promising source of lead compounds for pharmaceutical research and production. Therefore, profound exploration and conservation of *I. khasiana* are among the prime targets in research, as the plant is a critically endangered species and there is so much more to explore on its medicinal potentials.

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### Table 1: Preliminary phytochemical screening of three extracts of *Ilex khasiana* leaves.

| Phytochemical group | Petroleum Ether (IKP) | Chloroform (IKC) | Methanol (IKM) |
|---------------------|-----------------------|-----------------|----------------|
| Alkaloids           | +                     | -               | +              |
| Phytosterols        | -                     | +               | +              |
| Flavanoids          | -                     | -               | -              |
| Gums                | -                     | -               | -              |
| Reducing sugars     | -                     | +               | +              |
| Tannins             | -                     | -               | -              |
| Saponins            | -                     | +               | +              |
| Triterpenoids       | +                     | -               | +              |
| Amino acids         | -                     | -               | -              |
| Glycosides          | +                     | +               | +              |
| Carbohydrates       | -                     | +               | +              |

* + indicates presence; and – indicates the absence of a particular compound group.*
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