RESEARCH ARTICLE

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Investigation of the therapeutic properties of the leaves of *Eriobotrya japonica* (Thunb.) Lindl.

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ABSTRACT

Following previous studies that have confirmed the therapeutic potential of medicinal plants, scientists are now focusing on the potential of these medicinal plants in resolving issues associated with adverse drug reactions. Reports have been made on medicinal plants having fewer adverse drug reactions than synthetic drugs though a few medicinal plants have been approved by the Food and Drug Administration for therapeutic uses. The main aim of this research was to investigate the therapeutic properties of the leaves of *Eriobotrya japonica* (Thunb.) Lindl. The leaf constituents of *E. japonica* were extracted and quantified using maceration methods. The therapeutic potential of these phytochemicals was then investigated focusing on the antioxidant, anti-inflammatory, and antibacterial properties. The antioxidant properties were investigated using a newly proposed method called the Nitrocellulose Permanganometry assay. The anti-inflammatory and antibacterial assays were investigated using the albumin denaturation assay and broth dilution technique respectively. Alkaloids, flavonoids, and saponins showed anti-inflammatory activity by inhibiting albumin denaturation, with alkaloids having the most anti-inflammatory activity as compared to flavonoids and saponins. The relative intensities representing antioxidant potential showed that tannins had the highest antioxidant potency compared to the other tested phytochemicals. All the phytochemicals showed antibacterial activity against *Staphylococcus aureus* with cardiac glycosides being the most potent. Thus, *E. japonica* proved to have therapeutic potential as all the phytochemicals obtained from this plant showed anti-inflammatory, antioxidant, and antibacterial activity. Toxicity studies are therefore required to recommend the use of *E. japonica* as a natural remedy.

Key words: *Eriobotrya japonica*, therapeutic, alkaloids, flavonoids, saponins, cardiac glycosides, tannins, anti-inflammatory, antibacterial, antioxidant

Introduction

Most of the drugs in ancient times were derived from plants as plants consist of primary and secondary metabolites which are essential for their survival and self-defense, normal growth, development, and reproduction (Irchhaiya et al., 2015). These plant metabolites have been reported to perform various biological activities such as antimicrobial, anti-parasitic, enzyme inhibition, antitumor, and immune-suppressive (Thirumurugan et al., 2018). Thus, some plant metabolites are used as drugs, flavors, fragrances, insecticides, and as well as dyes (Petric et al., 2015).

Eriobotrya japonica (Thunb.) Lindl., commonly known as the Japanese Loquat, is a tree that is evergreen and belongs to the family *Rosaceae* (Liu et al., 2016; Shen et al., 2016). It has been reported to cure cough in Chinese folk medicine (Khatik et al., 2019). It has also been reported to contain tannins, flavonoids, coumarins, steroidal glycosides, alkaloids, proteins, quinones, saponins, and reducing sugars (Banno et al., 2005). The loquat tree extracts have been reported to be capable of counteracting inflammation, cancer, bacterial infection, aging, pain, allergy, and other health issues (Banno et al., 2005).

Numerous studies showed that the loquat fruit and leaf contained significant levels of flavonoids and phenolics (Banno et al., 2005), and both the methanol extract of loquat leaf and its component displayed strong antioxidant ability (Mojab et al., 2010). There is currently no research on the flavonoids, phenolics, and antioxidant activity of loquat leaves; instead, publications of the identification and analysis of the bioactive components in loquat flowers are mostly focused on triterpenoids and amygdalin (Heendeniya et al., 2018).

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Eriobotrya japonica contains phytochemicals that have antibacterial and antiviral properties. In a study by Silva et al. (2020), methanolic extract of the leaves of *E. japonica* recording high levels of chlorogenic, caffeic, and ellagic acids were able to inhibit the growth of *Staphylococcus aureus*. An *in vivo* study of the anti-oxidant properties of loquat leaves was reported in a study by Banno et al. (2005). In the study, the isolated terpenes used 12 of the 16 terpenes they isolated to evaluate their inhibitory effects on 12-*O*-tetradecanoylphorbol-13-acetate(TPA)-induced inflammation (1µg / ear) in mice. All of them had anti-inflammatory properties with a 50 % inhibitory dose (ID_{50}) of 0.03-0.43 mg per ear (Tolulope, 2017).

This present study focuses on investigating the antioxidant, anti-inflammatory, and antibacterial properties of alkaloids, saponins, flavonoids, tannins, and cardiac glycosides from the leaves of *E. japonica* to scientifically validate its use as a natural remedy.

Materials and Methods

Plant sample collection

The leaves of *E. japonica* were collected from a tree in Harare on the 3rd of October 2020, in Zimbabwe and were classified at the National Herbarium and Botanic Gardens of Zimbabwe by Mr. Christopher Chapano, a taxonomist. The voucher specimen number of the plant sample was BTBC-EJ-#01.

Plant sample preparation

The leaves were rinsed in water and were then dried in an oven set at 70°C for 48 hours. The leaves were ground to powder using a pestle and mortar. The powdered sample was weighed and stored at room temperature.

Phytochemical qualitative analyses

Qualitative determination of phytochemicals present in *E. japonica* was done using methods described by Mojab et al. (2010). For the alkaloids, about 1 g of the powdered sample was mixed with 1 ml of 10% ammonia solution and extracted with 5 ml ethanol for 10 min in a water bath at 40°C. The mixture was filtered through Whatman (№40) filter paper and the filtrate was concentrated by air drying. The residue was heated in a boiling water bath with 5 ml of 2N HCl. After cooling, the mixture was filtered, and to the filtrate few drops of Wagner's reagent were added and colour changes were observed and recorded. For flavonoids, isolation was done by heating approximately 1 g of the powdered sample with 5 ml methanol in a water bath at 40°C for 10 min. The mixture was filtered and the filtrate was then concentrated to a quarter of its original volume. The concentrated solution was treated with a few drops of concentrated HCI and a piece of magnesium turnings. The results were observed and recorded. For saponins, approximately 1 g of the dried sample was boiled in 20 ml distilled water. The extract was cooled and then filtered. The filtrate was shaken vigorously to froth and then allowed to stand for about 15 min. The samples were classified for saponin content as follows: no froth = negative, froth less than 1 cm = weakly positive, froth 1.2 cm = positive, and froth greater than 2 cm = strongly positive. For tannins, about 2 g of powdered sample was mixed in 50 ml methanol and the mixture was left to extract phytochemicals overnight. The next day the mixture was filtered and approximately 5 ml of this filtrate was allowed to react with a 5% $FeCl_3$ solution. The colour change was observed and recorded. For cardiac glycosides, about 2 g of powdered sample was mixed in 50 ml methanol and the mixture was left to extract phytochemicals overnight. The next day the mixture was filtered and 5 ml of this filtrate was treated with 2 ml glacial acetic acid containing 1 drop of 5% $FeCl_3$ solution. This solution was carefully transferred to the surface of 1 ml conc. H_2SO_4. The results were observed and recorded.

Phytochemical quantitative analyses

The quantitative analysis of all the phytochemicals from *E. japonica* leaves was done following the method by Kaur & Arora (2015). To quantify alkaloids, about 200 ml of 10% acetic acid in ethanol was added to 5 g of the powdered sample and stirred. The sample was covered and allowed to stand for 4 hours before it was filtered using Whatman paper (№1). The filtrate was concentrated in a water bath at 55°C to a quarter of its original volume. Concentrated ammonium hydroxide was added dropwise to the extract until the precipitation was complete. The whole solution was allowed to settle and collected precipitates were washed with dilute ammonium hydroxide and then filtered. The residue was dried, weighed, and stored in a closed container. The weight of alkaloids was recorded.

For flavonoids, about 10 g of powdered sample was extracted twice with 60 ml of 80% methanol at room temperature. The whole solution was filtered through Whatman filter paper №1, the filtrate was later transferred into watchglasses and evaporated to dryness to a constant weight in a water bath at 55°C. The weight of the flavonoids was recorded.

For saponins, about 20 g of each powdered sample was added to 100 ml of 20% aqueous ethanol and kept in a shaker for 30 min. The samples were heated in a water bath for four hours at 55°C. The mixture was then filtered and the residue was re-extracted with another 200 ml of 20% aqueous ethanol. The combined extracts were reduced to approximately 40 ml over a water bath. The resultant filtrate was put in a water bath at 90°C and was reduced to approximately 100 ml. About 40 ml was withdrawn from the concentrated sample and transferred into a separating funnel.

It was extracted twice with 20 ml diethyl ether. The ether layer was withdrawn from the funnel the aqueous layer was retained and about 60 ml of n-butanol was added to it. The extracts were washed thrice with 50% sodium chloride. The two layers that formed were separated by the funnel inserted into a crucible and left to evaporate to constant weight. The saponin content was calculated as a percentage of the initial weight of the sample taken.

Anti-inflammatory activity determination (Albumin Denaturation)

The anti-inflammatory assay of the alkaloids, flavonoids and saponins from leaves of *E. japonica* was carried out using the method described by Heendeniya et al. (2018). The diclofenac pills were ground to powder using a pestle and mortar. Approximately 50 mg of each phytochemical or drug was diluted with about 4 ml of the respective solvent to form a stock concentration of 12.5 mg/ml. Each stock was then serially diluted to make concentrations of 12.5, 6.25, 3.125, 1.5625, 0.78125 and 0.39062 mg/ml. The serial dilutions were made in test tubes such that the lowest volumes were 2 ml. PBS buffer (2.8 ml) at a pH of 6.3 was put in each of the 24 boiling tubes followed by approximately 2 ml of the phytochemical or the drug. About 0.2 ml of egg albumin was added to each tube. All the boiling tubes were heated in a water bath at 37°C for 15 min. The temperature was gradually increased up to 70°C for 5 min. The boiling tubes were allowed to cool to room temperature. The absorbance of each tube's contents was measured at 660 nm using the PBS buffer as a blank and the results were recorded.

Antioxidant activity determination (Nitrocellulose Redox Permanganometry)

The nitrocellulose Redox Permanganometry assay was done to investigate the antioxidant ability of alkaloids, flavonoids, tannins, cardiac glycosides, saponins, and diclofenac sodium B.P. This assay was carried out according to the method described by Homolak et al. (2022). About 1 mg of alkaloids, saponins, flavonoids, tannins, cardiac glycoside, and diclofenac sodium were measured and diluted with 1 ml of distilled water. Diclofenac sodium B.P. has antioxidant properties according to Boarescu et al. (2021). The protective cover of the nitrocellulose membrane was removed and the nitrocellulose membrane was placed on a clean laboratory surface. About 1 µl of each of the phytochemicals were placed onto the nitrocellulose membrane which was then left to dry out. The nitrocellulose membrane was dipped in potassium permanganate for about 30 seconds and then placed in distilled water to stop the reaction and increase contrast. The membrane was left to dry out and pictures of the membrane were taken. The intensity of each region where the extract or diclofenac sodium B.P. was placed was measured using FIJI (ImageJ) software. The photo of the nitrocellulose membrane was imported into FIJI. Each region of interest was selected using the rectangle selection tool, analysed and then integrated density was measured. The relative Integrated Density was calculated by the formula (Integrated density of Antioxidant – Integrated Density of blank).

Antibacterial activity determination (broth dilution assay)

Staphylococcus aureus was used as the test organism for investigating the antibacterial activity of phytochemicals from *E. japonica,* using the method explained previously by Mangoyi et al. (2012).

Resuscitation of Staphylococcus aureus

Staphylococcus aureus stock samples were resuscitated by inoculating 20 µl in 20 ml of sterile Luria Bertani broth and allowed to grow in a shaking incubator overnight at 37°C and 120 rpm. The overnight culture was streaked on Luria Bertani agar medium and the plates were incubated at 37°C in a non-shaking incubator. The plates were then stored at 4°C for later use.

Preparation of 1 × 10⁸ CFU of Staphylococcus aureus

A single and pure colony of *Staphylococcus aureus* was sub-cultured in 20 ml of Luria Bertani at 120 rpm and 37°C in a shaking incubator overnight. The overnight culture of *Staphylococcus aureus* was diluted with sterile Luria Bertani broth in order to reach an absorbance similar to that of the MacFarland standard solution which was in the range of 0.8-1.

Serial Dilutions of the plant phytochemicals and the antibiotics

A 96 well plate was used to make serial dilutions of tannins, saponins, flavonoids, cardiac glycosides, alkaloids, and cotrimoxazole and doxycycline solutions using the two-fold dilution method.

Minimum inhibitory concentration (MIC) determination

A sterile 96 well plate was used for the broth dilution technique. The first two columns of the well had 100 µl of Luria Bertani broth and 100 µl of extract or antibiotic. The third column up to the eighth column had 100 µl of extract or antibiotic solution in decreasing concentrations and about 100 µl of *Staphylococcus aureus* suspended in Luria Bertani broth. The ninth and tenth column had 200 µl of *Staphylococcus aureus* cells suspended in LB broth. The eleventh and twelfth columns had 200 µl of LB broth. All these components were agitated gently whilst adding each mixture into its respective well. The absorbance of samples in each well was measured using the Elisa Plate reader at 633 nm before incubation. The plate was closed and put in a box with a damp multi-wipe paper. The plate was incubated at 37°C overnight. The following day absorbance of each well

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was measured using the Elisa plate reader and the results were recorded.

Minimum Bactericidal Concentration (MBC) determination

The wells that showed no growth from the MIC determination assay were used for MBC determination. About 100 µl were withdrawn from wells that showed no growth and pipetted and spread on Luria Bertani agar. The plates were incubated at 37°C overnight and the results were recorded.

Results

Qualitative analysis

Qualitative analysis results showed that *E. japonica* leaves contain the phytochemicals saponins, flavonoids, cardiac glycosides, alkaloids, and tannins as shown in Table 1.

Quantitative analysis

Quantitative analysis was done for alkaloids, flavonoids, and saponins. The mass of these phytochemicals was determined and yield was determined as percentages. The percentage yields for alkaloids, flavonoids, and saponins are shown in Table 2. The results show that *E. japonica* leaves had the highest amount of flavonoids (5.33%) as compared to alkaloids (3.01%) and saponins (4.37%).

Anti-inflammatory activity

The percentage inhibition trends of albumin denaturation by alkaloids, flavonoids, saponins, and diclofenac sodium B.P. are shown in Figure 1. The results show that all tested samples had an inhibitory activity; however, their activity was very low as compared to the positive control diclofenac sodium B.P. As shown in Table 3, alkaloids had the highest inhibitory activity with the lowest IC_{50} of 42.16 mg/ml, followed by flavonoids and lastly saponins. Diclofenac sodium B.P. had the IC_{50} of 3.399 mg/ml.

Antioxidant activity

The nitrocellulose Redox Permanganometry assay results were digitalized and pictures are shown in Figures 2a and 2b. The nitrocellulose papers with potassium permanganate reacted with the phytochemicals and diclofenac sodium showing that all the tested samples had antioxidant activity.

The Relative Integrated densities for all the tested samples are shown in Table 4. These results show that all the tested samples had antioxidant activity; however, activity was lower than for the positive control for all samples.

Antibacterial Assay

Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration for each phytochemical was determined using absorbance differences

Table 1. *Observations and results from the qualitative analysis of the extracts from E. japonica.*

ID	Phytochemical	Observation	Result
1	Alkaloids	A reddish-brown precipitate was observed following addition of Wagner's Reagent	Positive
2	Flavonoids	The yellow solution turned colorless after addition of magnesium turnings	Positive
3	Saponins	A persistence foam of 0.9 cm was observed after 15 min	Weakly positive
4	Tannins	A green-blackish precipitate emerged after addition of 5 % $FeCl_3$ solution	Positive
5	Cardiac Glycosides	A reddish-brown layer was observed at the surface solution after transfer of 1 ml conc. H_2SO_4.	Positive

Table 2. *Percentage yield of alkaloids, flavonoids and saponins from the leaves of E. japonica.*

Metabolite	Yield (%)
Alkaloids	3.01 ± 0.009
Flavonoids	5.33 ± 0.03
Saponins	4.37 ± 0.05

Results are the average (± SD) of two separate percentage yields calculated

Table 3. *Inhibitory concentration at 50 % (IC_{50}) of alkaloids, flavonoids, saponins and diclofenac sodium B.P. on albumin denaturation.*

Phytochemical/Drug	IC_{50} (mg/ml)
Alkaloids	42.16
Flavonoids	3346
Saponins	6726
Diclofenac Sodium B.P.	3.999

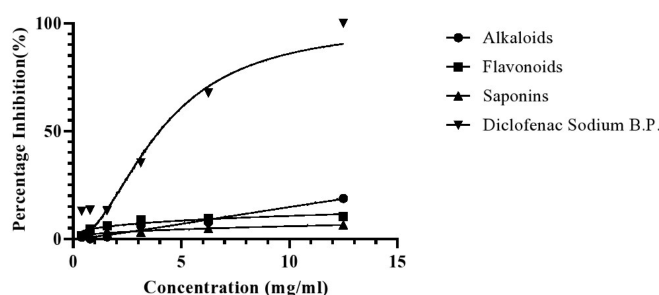


Figure 1. *Percentage Inhibition of albumin denaturation by different concentrations (mg/ml) of diclofenac sodium B.P., alkaloids, flavonoids and saponins from E. japonica leaves.*

obtained before and after the incubation of cells. The results show that cardiac glycosides were the most potent among all the tested phytochemicals as shown by their lowest MIC value (Figure 3). The positive controls cotrimoxazole and doxycycline had antibacterial activity but with low activity as compared to all the phytochemicals tested.

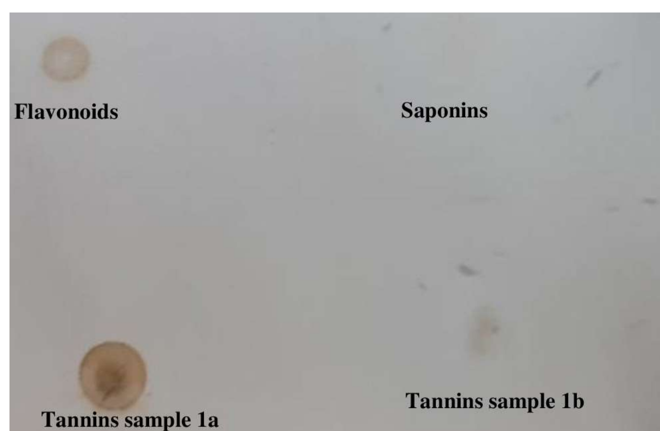
Minimum Bactericidal Concentration (MBC)

The results show that cardiac glycosides were the most potent among all tested phytochemicals as shown by their lowest value of MBC (Figure 4). The positive controls cotrimoxazole and doxycycline had high MBC values as compared to all the phytochemicals tested.

Table 4. *The integrated densities and relative integrated densities of phytochemicals from leaves of E. japonica and diclofenac sodium B.P.*

Phytochemical	Integrated density	Relative Integrated density
Blank	508330	0
Alkaloids	860230	351900
Flavonoids	984045	475715
Saponins	553404	45074
Cardiac glycosides	1089740	581410
Tannins	1138694	630364
Tannins	750923	242593
Diclofenac Sodium	1271664	763334

a



b

Figure 2: a) *Alkaloids, flavonoids, Cardiac glycosides and diclofenac Sodium B.P. Nitrocellulose Redox Permanganomertry digitalised results;* **b)** *Flavonoids, tannins and saponins Nitrocellulose Redox Permanganometry digitalized results.*

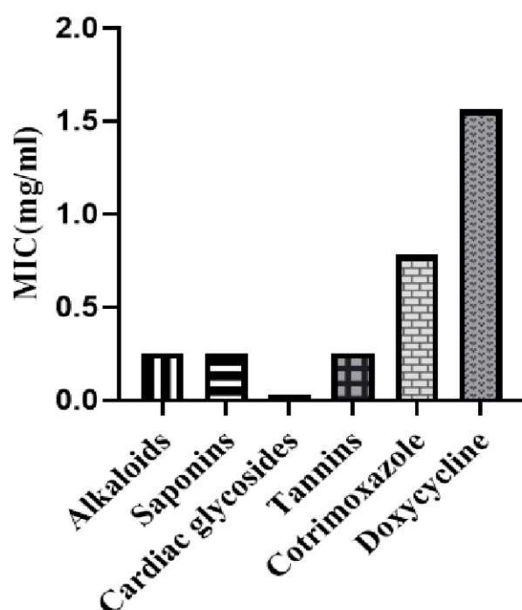


Figure 3. *The MIC values of phytochemicals from the leaves of E. japonica, Cotrimoxazole and Doxycycline.*

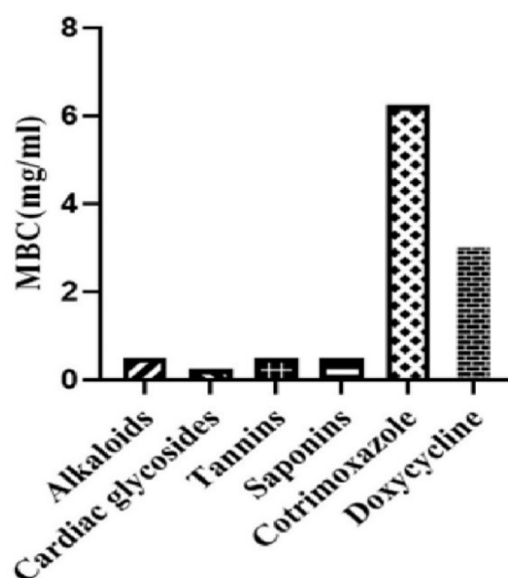


Figure 4. *The MBC values of phytochemicals from the leaves of E. japonica and antibiotics (Cotrimoxazole and Doxycycline).*

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Discussion

In the present study phytochemical qualitative assays were carried out to determine the presence of alkaloids, tannins, saponins, cardiac glycosides, and flavonoids in the leaves of *E. japonica*. These phytochemicals were utilized in antibacterial, antioxidant, and anti-inflammatory assays to determine their therapeutic properties.

The anti-inflammatory potential of the leaves of *E. japonica* was assessed in this study using the induced egg albumin denaturation bio-assay. The results suggested anti-inflammatory inhibition properties of alkaloids, flavonoids, and saponins from *E. japonica* with alkaloids having the highest anti-inflammatory potential with an IC_{50} of 42.16 mg/ml, followed by flavonoids and saponins. The reference drug diclofenac sodium B.P. also showed anti-inflammatory activity, however, the IC_{50} obtained (3.999 mg/ml) was different from the IC_{50} reported in the previous study of 170 400 mg/ml (Heendeniya et al., 2018). The differences may be due to the differences in bioavailability of different brands of the drug used (Al-Ameri et al., 2012). The leaves of *E. japonica* were previously reported to contain methyl ursolate and a terpenoid saponin ursolic acid lactone which have anti-inflammatory properties (Bhutia, 2020; Dhiman et al., 2022).

Antioxidant properties of the selected phytochemicals from leaves of *E. japonica* were assessed using the Nitrocellulose redox Permanganometry assay in this study. The ImageJ application was then used to quantitate the amount of manganese dioxide trapped in the nitrocellulose membrane following reduction of potassium permanganate by the extracted phytochemicals from the leaves of *E. japonica*. Amongst the plugins of the application were the ones that measured the intensity of manganese dioxide and such information was used to assess the antioxidant potential of the extracts from the leaves of *E. japonica*. The reductive capacity of the sample was measured by its capacity to reduce potassium permanganate to manganese dioxide. The observed results as explained by the obtained Relative Integrated densities in Table 4, showed that at the concentration of about 1 mg/ml, the tannins had the highest antioxidant potency followed by cardiac glycosides, flavonoids, alkaloids, and saponins. However, all the phytochemicals had much lower antioxidant activity as compared to the diclofenac sodium pill of the same concentration. Generally, the antioxidant potency of the saponins was significantly low as compared to all the other phytochemicals from leaves of *E. japonica* and diclofenac sodium.

A study by Maher et al. (2015) predicted that flavonoids and phenolic compounds from leaves of *E. japonica* have antioxidant properties, and among phenolic compounds are tannins. This observation coincides with the present study as tannins and flavonoids were found to have antioxidant activity. Flavonoids and tannins are phenolic compounds that are known to be antioxidants. They have hydroxyl groups which are good hydrogen donors and react with potassium permanganate giving manganese dioxide and potassium hydroxide (Pereira et al., 2009).

The leaves of *E. japonica* have been found to have phytochemicals that have antibacterial properties in this study. These results are in agreement with the study conducted by Zhou et al. (2007) who stated that the MIC of extracts from *E. japonica* was between 0.06 and 1.25 mg/ml whereas MBC was between 0.13-4 mg/ml.

Thus, the findings from this study showed that all the tested phytochemicals from the leaves of *E. japonica* may have antibacterial properties that are more potent than doxycycline and cotrimoxazole as explained by their low MIC values.

Conclusion

All the phytochemicals isolated from *E. japonica* showed some therapeutic effects. Alkaloids had the most anti-inflammatory potential; tannins had the most antioxidant potency and cardiac glycosides had the most antibacterial activity against *Staphylococcus aureus*. Thus, the leaves of *E. japonica* may be recommended for use as a natural remedy.

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