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In vitro Antioxidant and effects of ethanol extract of *Syzygium aromaticum* on Gastrointestinal smooth muscle

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ABSTRACT

Background: *Syzygium aromaticum* (Family: Myrtaceae) has long been used to treat a range of gastrointestinal conditions, including chronic diarrhea, flatulent colic, and gastric irritation. Due to the numbing action, fried *Syzygium aromaticum* flower buds combined with honey have been used for years to prevent vomiting. The objective of this study was to investigate the phytochemical constituents, *in vitro* antioxidant and the effect of ethanol extract of *Syzygium aromaticum* on isolated rabbit jejunum.

Method: Standard techniques were used to determine the phytochemical screening of ethanol extract of *Syzygium aromaticum* (EESA) flower buds. Total antioxidant contents (TAC) and 1,1-diphenyl-1-picrylhydrazyl (DPPH) scavenging were the *in vitro* antioxidant activities evaluated against EESA utilizing conventional methods, at concentrations of 200, 400, 600, 800 and 1000 µg/mL. Atropine (0.1 µg/mL) and acetylcholine (0.1 µg/mL) were employed as a standard anticholinergic and agonist respectively, in rabbit jejunum to screen the extract utilizing isolated rabbit jejunum. The rabbit jejunum's spontaneous amplitude and frequency were measured following the administration of an ethanol extract from *Syzygium aromaticum,* at the concentrations' of (0.2, 0.4 and 0.6 mg/mL).

Results: Terpenoid, alkaloids, flavonoids, tannins, phenols, and saponins were detected by phytochemical screening. Against total antioxidant substances, the EESA's activity was 3.11±0.60 mg ASCE/g. The scavenging activity of EESA 250 µg/mL against the DPPH radical was lower than that of other concentrations, with an IC_{50} value of 212.87 (µg/mL). When compared to the relaxation response, all extract concentrations inhibited acetylcholine-induced intestinal motility in a manner that was strikingly similar to that of a common anticholinergic agent (Atropine). This suggests that our extract may have worked by binding and blocking muscarinic receptors in the gastrointestinal tract's smooth muscles, hence inhibiting intestinal contractions mediated by the parasympathetic nervous system.

Conclusion: Accordingly, our findings imply that the ethanol extract of *Syzygium aromaticum* contains significant levels of phenolic compounds, which demonstrated strong antioxidant properties and may aid in halting the development of different oxidative stressors. Furthermore, EESA's active ingredients have potent anticholinergic qualities, which promises well for the creation of a novel anticholinergic medication with few adverse effects that could help treat a range of intestinal disorders.

1. Introduction

The autonomic nervous system's sympathetic (adrenergic) and parasympathetic (cholinergic) arms interact to balance the majority of bodily physiological processes; excessive or insufficient activity of either arm causes physiological disorders[1,2]. Asthma, incontinence, gastrointestinal spasms, gastritis, peptic ulcers, diarrhea, sleeplessness, muscle cramps, chronic obstructive pulmonary sickness, and motion sickness are among the conditions brought on by an overactive parasympathetic arm[3,4]. According to

Jackquelyn[3], these illnesses are caused by excessive involuntary muscle action linked to an excess of acetylcholine, a neurotransmitter that mediates cholinergic activities.

Cholinergic blockers prevent the neurotransmitter; acetylcholine from acting in the parasympathetic discharge and hence reduce cholinergic nerve impulses. Anticholinergic drugs help treat conditions brought on by excessive parasympathetic activity, such as those affecting the gastrointestinal tract, by decreasing the effects of acetylcholine by competitive inhibition[5]. Due to the numerous adverse effects of current anticholinergic medications, such as, scopolamine and atropine, caused dry mouth, blurred vision, constipation, urinary retention, dizziness and drowsiness, tachycardia and flushing and dry skin, as well as factors like cost, custom, culture, and efficacy, herbal medicine has gained popularity, and as a result, the use of plants for medical purposes is still expanding throughout the world today[6].

Syzygium aromaticum is one of such plants that is being exploited and is reported to be used traditionally for its anticholinergic property[7]. The tree *Syzygium aromaticum* (L.) Merr. & Perr., is a member of the Myrtaceae family. Also known as Cloves, native of Moluccas, in Indonesia, the fragrant flower buds, are used as a spice in many different contexts. Commercial clove harvesting takes place in a number of African countries, including Madagascar, Seychelles, Tanzania, Indonesia, India, Pakistan, and Sri Lanka. Eugenol, β-caryophyllene, eugenol, eugenin, and oleanolic acid are among the many bioactive substances they contain[8]. Historically, *Syzygium aromaticum* has been linked to strengthening of the immune system and encouraging resistance to disease. The flower buds of the *Syzygium aromaticum* plant are used to treat a variety of conditions, including dental and oral problems[9,10]. Additionally, it is used as an antiemetic to stop vomiting and to treat indigestion, flatulence, cough, diarrhea, stomach distension, and gastrointestinal spasms; it also relieves pain, stimulates the nerves, and induces uterine contractions[11].

This fragrant plant's essential oil serves as a dietary antioxidant that is thought to offer protection against a number of ailments brought on by free radicals in addition to being a fragrance and flavoring ingredient[9,12]. According to earlier research, *Syzygium aromaticum's* antioxidant effect is linked to the synergistic interaction of phenolic components and secondary metabolites in the oil[13]. Additionally, a number of studies have shown that most synthetic antioxidants are toxic and carcinogenic, meaning they cannot shield the body from attacks by free radical oxidation if taken in excess.

Thus, the use of natural antioxidants may help eliminate free radicals without interfering with the body's defense mechanism[13]. This study investigates the phytochemical components, *in vitro* antioxidant, and impact of ethanol extract of *Syzygium aromaticum* flower bud on isolated rabbit jejunum.

Materials and methods

Plant material

Plant collection and identification

Syzygium aromaticum flower buds were bought at the Sagamu local market in March of the year 2024, Ogun State, Nigeria. The samples were kept in the Department of Pharmacognosy's herbarium at Olabisi Onabanjo University (OOU), Sagamu, Ogun State, Nigeria, for future use after the buds were identified and authenticated by Mr. Adeoti, O.A. of Department of Pharmacognosy at Olabisi Onabanjo University with voucher number: PHS/OOU/339

Extraction of plant material

After being cleansed and let to air dry for seven days, the *Syzygium aromaticum* flower buds were grounded into a coarse powder. A Soxhlet extractor was used to extract the powder (100 g) with 1 L of ethanol (80%). A rotary evaporator was then used to concentrate the extract to dryness in vacuo, yielding an average of 9.5% (w/w). The dried extract was stored in a refrigerator at 4^0C and dissolved in distilled water prior to each experiment.

Preliminary Phytochemical screening

For both qualitative and quantitative screenings, the established protocol by Trease and Evans[14] and De Silva et al.[15], was used to test the phytochemical contents of the ethanolic extract of *Syzygium aromaticum*flower buds

Antioxidant Activities

Total antioxidant contents (TAC)

The phosphomolybdenum method was used to evaluate the overall antioxidant capability of the *Syzygium aromaticum* ethanol extract, in accordance with the procedure previously described by Phatak and Hendre[16]. The reduction of Molybdenum (Mo) (VI) to Mo (V) by the extract, followed by the formation of a green phosphate/Mo(V) complex at an acidic pH, provides the

basis for the test. 20 mL of distilled water, 1 mL of each of 0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate, made up to 50 mL distilled water was used to produce the molybdate reagent solution.

Each of the five test tubes held 3 mL of the molybdate reagent solution, and the ethanol extract of *Syzygium aromaticum* was added in an equal volume after being serially diluted to different concentrations (200, 400, 600, 800, and 1000 µg/mL). The reaction solution's absorbance was measured at 695 nm after the test tubes containing it were incubated for 90 minutes at 95^0C and then let to stand at room temperature for 20 to 30 minutes. Ascorbic acid Equivalent (ASCE) milligrams per gram of dried extract was the unit of measurement for the antioxidant activity.

1,1-diphenyl-1-picrylhydrazyl (DPPH) free radical-scavenging activity evaluation

Preparation of DPPH solution

To make the DPPH solution, the crystalline solid of roughly 0.1 mM DPPH was put in a test tube and gradually dissolved in an organic solvent (methanol). In a test tube, 3 mL of *Syzygium aromaticum* ethanol extract, standard (ascorbic acid) {ASA}, and control (an equivalent amount of methanol but without the test component) were taken at different concentrations (50, 100, 150, 200, and 250 µg/mL)[17]. One milliliter of the DPPH solution was progressively added to the *Syzygium aromaticum* extract. A spectrophotometer was used to measure the absorbance at 517 nm after the concentrate solution was shaken and left at room temperature in the dark for approximately half an hour.

The substance's ability to scavenge free radicals is shown by a decrease in absorbance. The following formula was used to determine the DPPH radical's % inhibition:

$$\% \text{ Inhibition} = \frac{(\text{Absorbance of Control} - \text{Absorbance of Sample})}{\text{Absorbance of Control}} \times 100$$

The IC_{50} was used to express the antioxidant activity. The quantity in µg/mL of the portion that inhibits 50% of DPPH radicals is known as the IC_{50} value.

Fourier-Transform Infrared Spectroscopy (FT-IR) Analysis

The plant's FT-IR analysis was conducted using methods that had been validated by Liu and Kim et al.[18] and Bolade et al.[19]. The Agilent Cary 630 FTIR spectrometer was used to investigate the *Syzygium aromaticum* extract (1% w/w). Microlab PC software and an attenuated total reflectance (ATR) sample unit with an 8 $cm^{-}1$ resolution and a scan range of 4000 cm^{-1} to 400 cm^{-1} were included with the infrared spectrometer.

Animal and Tissue Preparation

Amos et al.[20] described a modified method that was applied. Rabbits (1.6-2.4kg) acquired from the animal production unit of the Department of Physiology, College of Medicine, University of Lagos, Idi Araba, Lagos, Nigeria, were used for the study. They were fed with standard feed, with water *ad libitum,* and given a week to acclimate in the animal transit room normal humidity, temperature and lighting conditions. The guidelines set forth by the Institutional Animal Ethics Committees (IAEC) were adhered to. This work complied with the WMA Statement on the Use of Animals in Biomedical Research, the EU regulations (Directive 2010/63/EU) for the design and analysis of experiments in pharmacological care, and/or the advice of an internationally recognized authority. The Animal Care and Use Research Ethics Committee (CMUL/ACUREC) of the University of Lagos College of Medicine gave its ethical approval for the study's use of animals. Permission for the study was granted under CMUL/ACUREC/02/25/1758.

Prior to the experiments, the rabbits had unrestricted access to water despite their overnight fast. Rabbits were sacrificed by a knock on the head and exsanguinated the next day. The gastrointestinal tract was revealed by dissecting the abdomen, and the small intestine was meticulously separated, cleansed of its contents, and cleared of mesentery. To get the jejunum, a cut was then made around 15 cm from the ileocecal junction toward the top portion of the gastrointestinal system. In a Petri plate with Tyrodes solution of the following composition, the jejunum was promptly placed: 8.0 grams of chloride potassium, 0.05g of sodium phosphate, 0.2g of calcium chloride, 1.0g of glucose, 0.2g of sodium chloride, 0.1g of magnesium chloride, and 1.0g of sodium carbonate. Special care was observed while treating the jejunum and the use of forceps was carefully avoided. Tyrodes solution was kept under continual aeration with carbogen gas (95% O_2+ 5% CO_2). Four centimeters length segments of the jejunum were sliced and hung vertically in 10 mL tissue baths containing Tyrodes solution. Additionally, carbogen was used to bubble the bath solution, which was kept at 37ºC. A force-displacement transducer attached to the PowerLab Model 26T and lab chart software (Lab chart 8) were used to record the intestinal contractile responses in order to measure isometric contraction. Before any agonist, antagonist, or

extract was added, the tissues were given 30 minutes to equilibrate under 1g (0.5N) strain, carried out in triplicates.

Statistical Evaluation

Data were presented as mean ± standard error of mean (SEM). The IC_{50} values were determined by linear regression analysis. One-way ANOVA and Dunnett's post-hoc tests for multiple comparisons were used to compare the results. The statistical analysis was conducted using Graph Pad Prism 6. A P-value of 0.05 or above was used to confirm the level of significant *(P*<0.05) .

Results

Results of phytochemical screening of ethanol extract of *Syzygium aromaticum* (EESA)

Tannins, phenols, alkaloids, and flavonoids were found in high concentrations in ethanol extract of *Syzygium aromaticum* according to the phytochemical screening; the corresponding amount is shown in % below in Table 1.

Table 1: Qualitative and Quantitative Phytochemical Screening Results

Qualitative screening	*Syzygium aromaticum*	Quantitative Screening (%w/w)
Tannin	++ve	1.38±0.010
Phenol	++ve	1.33±0.006
Alkaloid	++ve	2.70±0.003
Saponin	+ve	0.45±0.002
Flavonoid	++ve	5.57±0.048
Terpenoid	+ve	1.10±0.001

Interpretations

+ve = present

++ve = abundantly present

Total antioxidant content (TAC) of ethanol extract of *Syzygium aromaticum* (EESA)

As indicated in Table 2, the results demonstrated varying levels of antioxidant activity at various doses. Antioxidant activity increased with concentration at all concentrations. The maximum overall antioxidant level, however, was found to be in the 1000 µg/mL concentration which is comparable to 3.95 mg/g ascorbic acid equivalent. At various doses, the average TAC of EESA was calculated to be 3.11 ± 0.60 mg of Ascorbic acid equivalent in gram (ASAE/g).

Table 2: Total antioxidant content (TAC) of ethanol extract of *Syzygium aromaticum*

EESA Concentration (µg/mL)	200	400	600	800	1000	Mean ±SEM
TAC (mg/ ASAE/g)	2.30	2.91	3.14	3.23	3.95	3.11±0.60

The Mean value in the table is expressed as the Mean ± SEM

DPPH scavenging activity (% inhibition) by ascorbic acid (ASA) and ethanol extract of *Syzygium aromaticum* (EESA)

Table 3 displays the results of the DPPH radical scavenging activity of various doses of *Syzygium aromaticum* ethanol extract and the reference ascorbic acid. Every EESA concentration that was examined showed dose-dependent in vitro DPPH radical scavenging activity. Similarly, when concentrations rose, the standard (ascorbic acid) showed an increase in DPPH radical scavenging capabilities. Ascorbic acid's IC_{50} value was 20.5µg/mL, while EESA was 212.87µg/mL (Table 3 and Figure 1). DPPH assays allow for the comparison of anti-oxidant activities across different crude drugs or extracts, aiding in the identification of those with potent anti-oxidant properties.

Table 3: *In vitro* DPPH scavenging activity (% inhibition) by ascorbic acid and ethanol extract of *Syzygium aromaticum*

DPPH scavenging activity (% inhibition)

EESA Concentration (µg/mL)	**50**	**100**	**150**	**200**	**250**	**Mean ±SEM**	**IC_{50}**
% Inhibition of DPPH by EESA	36.29	38.62	40.96	40.98	44.22	40.21±2.96	212.87 (µg/mL)
% Inhibition of DPPH by ASA	53.80	55.13	64.64	70.72	72.24	63.31±8.57	20.5 (µg/mL)

The Mean value in the table is expressed as the Mean ± SEM

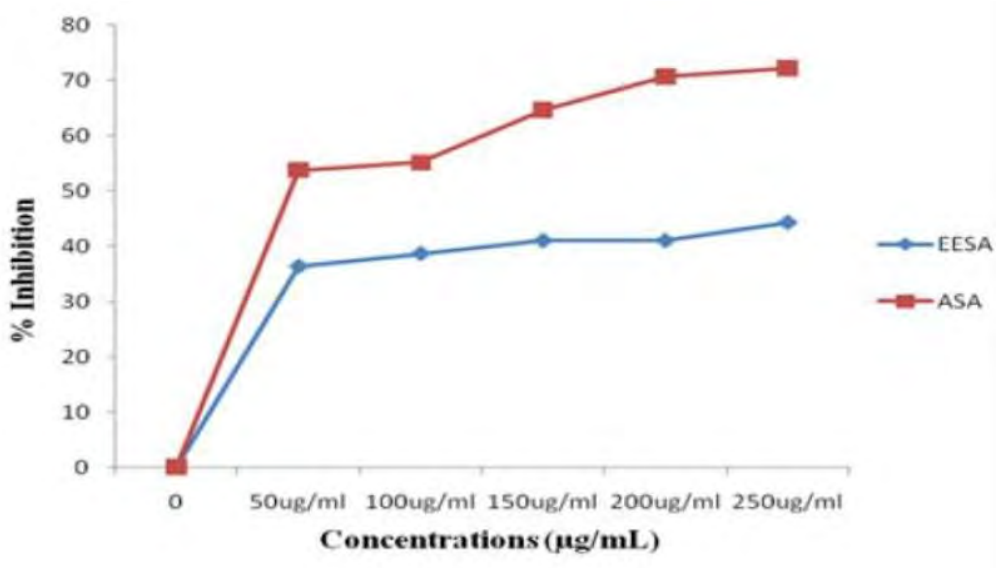

Figure 1: Plot of DPPH radical scavenging percentage between ascorbic acid and ethanol extract of *Syzygium aromaticum*.

FT-IR spectrum of methanol extract of ethanol extract of *Syzygium aromaticum*

Figure 2 showed that the spectrum of ethanol extract of *Syzygium aromaticum* peaks and wavelengths. The prominent peaks shown in figure 2 are, 2922.91 cm^{-1} 2863.61cm^{-1}, 1744.77 cm^{-1}, 1460.22 cm^{-1}, 1161.05 cm^{-1} and 721.63 cm^{-1} wavelength of ethanol extract of *Syzygium aromaticum*

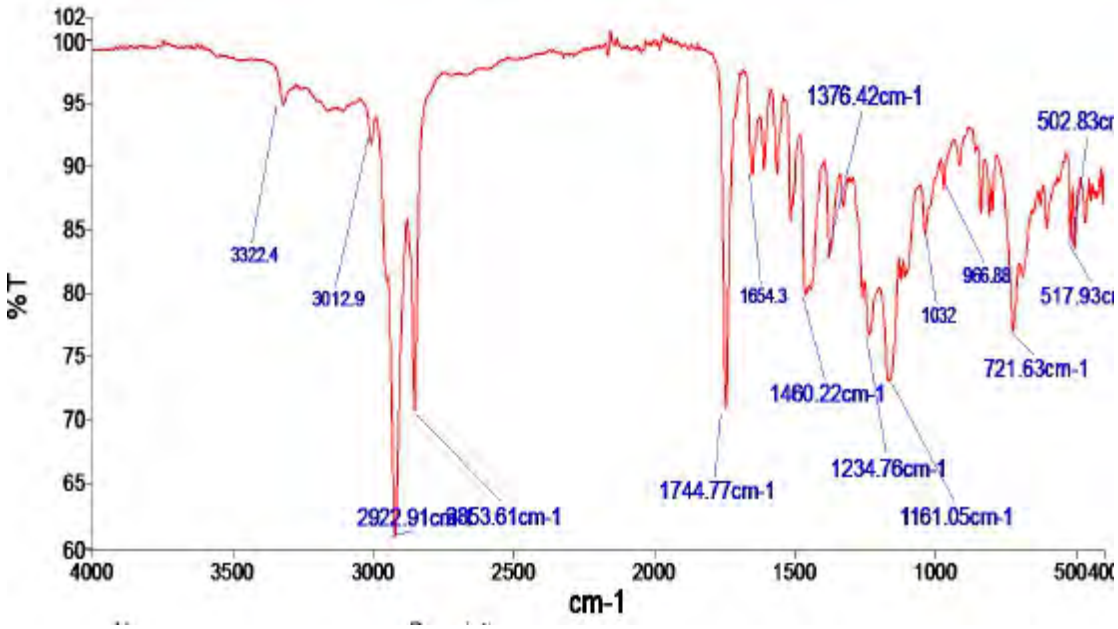

Figure 2: Plot of FT-IR spectrum of methanol extract of ethanol extract of *Syzygium aromaticum*.

Effect of the degree of response of the Extract, Acetylcholine, Atropine and Atropine + Acetylcholine +Extract

Syzygium aromaticum ethanol extract relaxed the rabbit jejunum in a concentration-dependent manner. On the other hand, acetylcholine caused a concentration-dependent constriction of the rabbit jejunum. Additionally, by causing a concentration dependent blockade of the constriction of the jejunum induced by acetylcholine, ethanol extract of *Syzygium aromaticum* demonstrated a comparable outcome to atropine. In the meantime, atropine and *Syzygium aromaticum* ethanol extract administered together after acetylcholine administration counteracted acetylcholine-induced contractions more effectively than either component alone (Figures 3, 4, 5,6 and Table 4).

Table 4: The degree of response of the Extract, Acetylcholine, Atropine and Atropine + Acetylcholine +Extract

	Contractile Responses (Newton)			
Concentration in mg/ml	**EXTRACT (EXT)**	**Acetylcholine (ACH)**	**Atropine (ATR)**	**ATR+ACH+EXT**
0.2	0.25±2.1	1.77±2.1	0.25±0.1	0.38±0.8
0.4	0.24±0.7	1.18±1.1	0.23±0.3	0.36±1.9
0.6	0.18±1.5	0.71±3.5	0.17±1.7	0.33±0.33

The Mean value in the table is expressed as the Mean ± SEM, n=3.

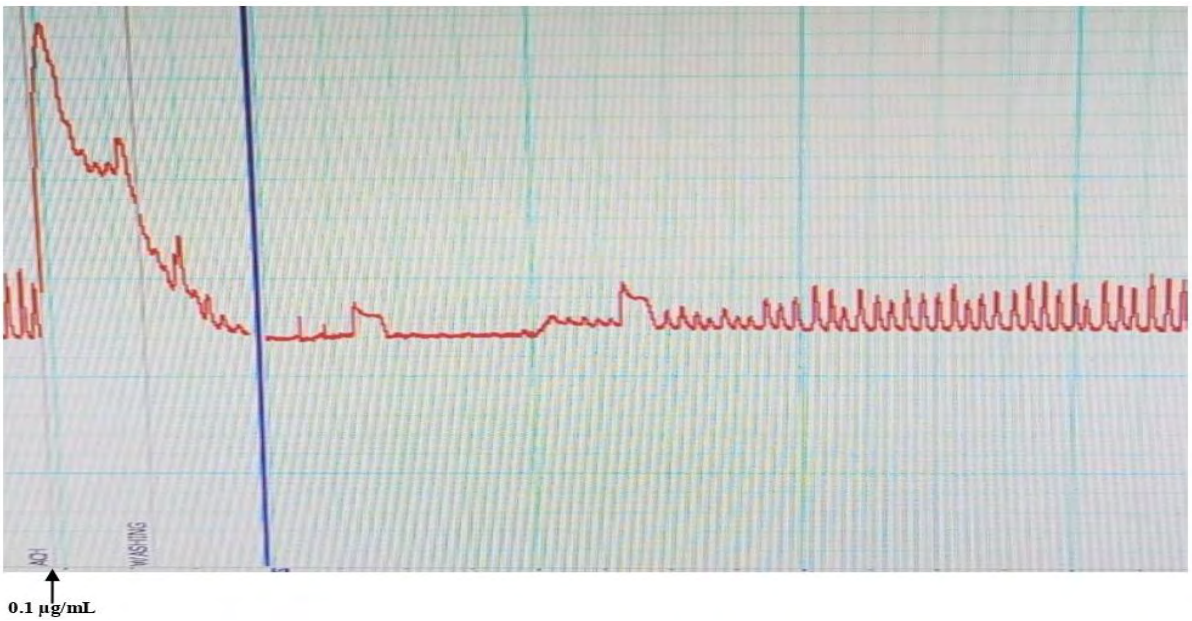

Figure 2: The contractile effects of Acetylcholine (ACH) on rabbit jejunum

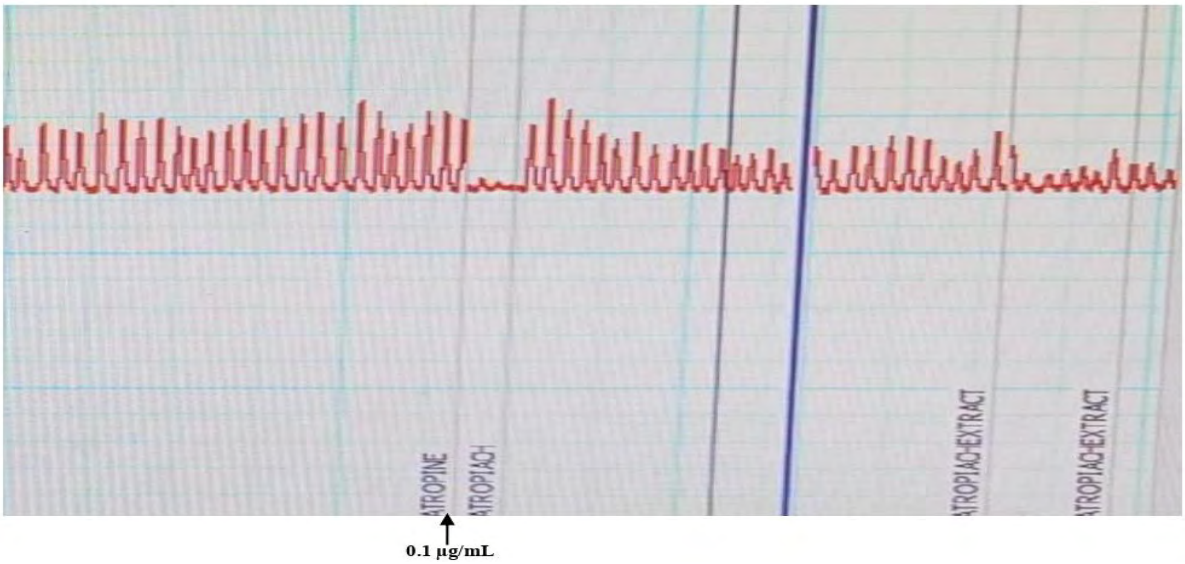

Figure 3:The relaxant effects of Aropine on rabbit jejunum

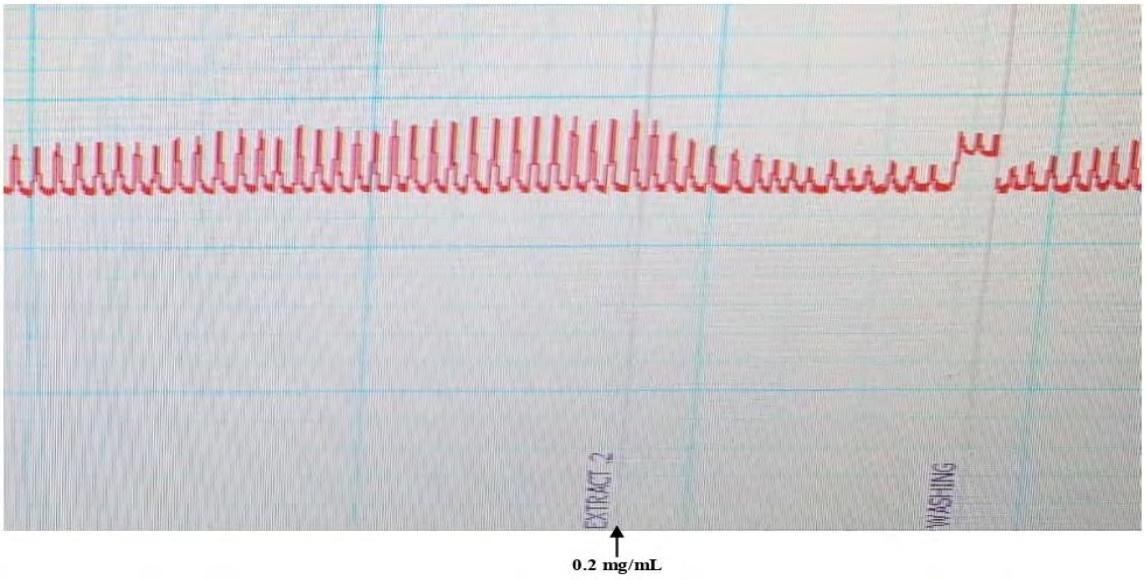

Figure 4: The relaxant effects of ethanol extract of *Syzygium aromaticum* on rabbit jejunum

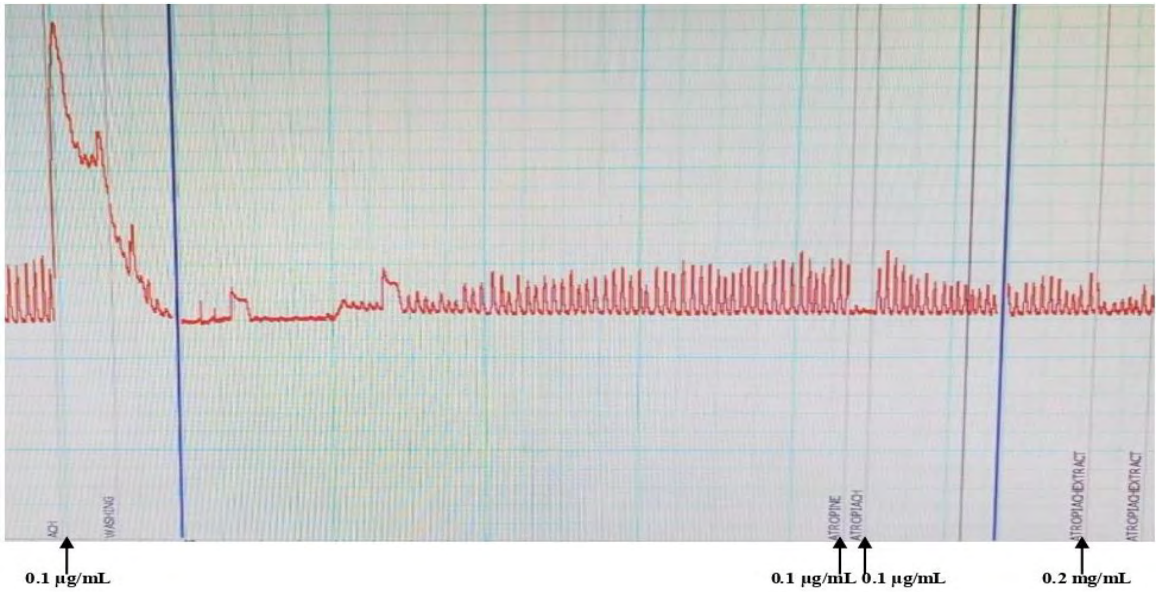

Figure 5: The contraction by Acetylcholine (ACH) and relaxant effects by a combination of Atropine and ethanol extract of *Syzygium aromaticum* on rabbit jejunum

Discussion

Oyinloye et al.[21] claim that the medical benefit of medicinal plants is attributed to their secondary metabolites, or phytochemicals, and other chemical constituents. *Syzygium aromaticum* flower bud's antioxidative properties and usefulness as a herbal remedy are due to its rich composition of phenolic chemicals[22]. Furthermore, flavonoids have also been demonstrated to demonstrate their actions through effects on membrane permeability and by inhibiting membrane-bound enzymes such as ATPase and phospholipase A2, which may account for the good antioxidative activity of the ethanol extract of *Syzygium aromaticum* buds in our current study[22].

Total antioxidant contents result showed increasing antioxidant activity with increasing concentration. Nevertheless, the maximum total antioxidant level (3.95 mg of ASAE/g) was seen in the EESA 1000 mg/kg. There was less antioxidant activity in EESA 200 mg/kg (2,30 mg of ASAE/g). The study's results corroborated those of Beatrice et al.[23], who asserted that the concentration of antioxidant components in a plant extract determines its antioxidant effectiveness. Our study found that the ethanol extract of *Syzygium aromaticum* has concentration dependent DPPH radical scavenging activity, which is similar to our previous research period[22].

The extract's inhibitory concentration that prevented 50% of the production of DPPH radicals was used to express the antioxidant activity of the *Syzygium aromaticum* ethanol exract. The standard (positive control) was ascorbic acid; the ethanol extract of *Syzygium aromaticum* and the standard, gave IC_{50} values of 20.5 and 212.87µg/mL, respectively. The present study supports earlier findings by Oyinloye et al.[21], who claimed that flavonoids and related polyphenols significantly contribute to medicinal plants' antioxidant activity.

Fourier Transform Infrared Spectroscopy (FTIR) stands as one of the extensively utilized techniques for identifying functional groups within bioactive molecules present in compounds. The FTIR spectrum of *Syzygium aromaticum* extract exhibited prominent peaks at 2922.91 cm^{-1} 2863.61cm^{-1}, 1744.77 cm^{-1}, 1460.22 cm^{-1}, 1161.05 cm^{-1} and 721.63 cm^{-1}, indicating characteristic stretches such as C–H (~2900 cm^{-1}), C—O (~1700 cm^{-1}), and C–O (~1100 cm^{-1}).

Acetylcholine, like other cholinergic agonists, producedcontractile responses when administered *in vitro,* as exhibited in the current investigation. Muscarinic receptors found in the gastrointestinal tract's smooth muscles are activated by acetylcholine, which causes smooth muscle to contract[24]. In addition, the isolated rabbit jejunum is subjected to a constant relaxing effect due to atropine's intrinsic pendular motions. The ethanol extract of *Syzygium aromaticum* showed a dose-dependent suppression of intestinal contractions generated by acetylcholine, similar to that of atropine. These findings imply that the ethanol extract of *Syzygium aromaticum* may have produced these effects by binding to the muscarinic receptors found in the intestinal smooth muscles, blocking acetylcholine's action, and preventing intestinal peristaltic contractions. Flavonoids are known to suppress minor intestine transients and contractions brought on by spasmogen[20]. Such characteristics could be the cause of the pharmacological effects that have been reported. The findings offered a helpful guide for identifying active ingredients in *Syzygium aromaticum*.

Conclusion

It is therefore logical to draw the conclusion that the ethanol extract of *Syzygium aromaticum* flower bud's ability to inhibit acetylcholine-induced contractions in *in vitro* experiments, provides evidence that the extract contains compounds with strong anticholinergic qualities and could be useful in the development of novel anticholinergic medications with few adverse effects for the treatment of gastrointestinal spasms.

Ethical statement

Institutional Animal Ethics Committee's requirements (IAEC) were adhered to. This work complied with the WMA Statement on the Use of Animals in Biomedical Research, the EU regulations (Directive 2010/63/EU) for the design and analysis of experiments in pharmacological care, and/or the advice of an internationally recognized authority.

Declaration of competing interest

Authors declares no conflict of interest. Data availability Data will be made available on request.

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