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Research Article

# Phytochemical Screening, *In vitro* Antioxidant Activities and Zebrafish Embryotoxicity of *Abelmoschus esculentus* Extracts

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## ABSTRACT

**Background:** *Abelmoschus esculentus* (L.) Moench (*A. esculentus*) commonly known as okra is being used as a medicinal plant traditionally, due to its phytochemical content that exhibits significant biological activities. **Objective:** The present study was undertaken to determine phytochemicals, antioxidant activity and embryotoxic effects of hexane extract (HE), chloroform extract (CE), methanol extract (ME), and aqueous extract (AE) of *A. esculentus* cultivated in Malaysia. **Materials and Methods:** *A. esculentus* extracts were screened for the phytochemicals while *in vitro* antioxidant activities were evaluated by performing 1,1-diphenyl-2-picrylhidrazyl (DPPH) assay and reducing power assay. Meanwhile, the embryotoxicity were accessed by exposing zebrafish embryos to the extracts and developmental endpoint recorded with median lethal concentration ($LC_{50}$). **Results:** The phytochemical screening showed the presence of flavonoids, tannins, phenols, saponins, anthraquinones, alkaloids and reducing sugars. The highest phenolic content was obtained in the CE at 143.85 µg (GAE)/mg extract. Whereas the HE depicted the highest flavonoid content at 63.76 µg (QE)/mg extract. At 1000 µg/mL, ME showed the highest free radical scavenging activity at 28.86 % and reducing power at 0.271, in both DPPH and reducing power assay, respectively. While the CE presented a significant embryotoxic effect on zebrafish with the $LC_{50}$ at 236.07 µg/mL. The morphological malformation of embryos, scoliosis and pericardial oedema were observed at 500 µg/mL of HE and ME treatment. **Conclusion:** As a result, with various ranges of phytochemical compounds, antioxidant capacities and lower toxic effects of *A. esculentus* extracts may be efficient in reducing the inflammation precursors that drive chronic inflammatory illnesses.

**Key words:** *Abelmoschus esculentus*, Antioxidant capacity, Phytochemicals, Zebrafish embryotoxicity.

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## INTRODUCTION

Nature has provided us with a plethora of medicinal plants. These medicinal plants are widely utilised across the world as a source of therapeutic agents for the prevention and treatment of various diseases.[1] Malaysia is one of the world's 12 megadiverse countries, with an estimation of 2000 medicinal plant species with health-promoting properties.[2] Nowadays, patients prefer plant medicines over conventional medicines as they minimise the disease effects and improve the life quality.[3] Multiple phytochemical constituents in medicinal plants are responsible for their medicinal and pharmacological impact that can produce the desired physiological response in the body.[1,4]

*Abelmoschus esculentus* (L.) Moench (*A. esculentus*) is among the regularly used species of a flowering plant belonging to the *Malvaceae* family and it is commonly known as okra.[5,6] The origins of *A. esculentus* are disputed, with proponents claiming whether it originated from South Asia, Ethiopia or West Africa. This commercially significant vegetable crop is farmed all over the world in tropical, subtropical and mild temperate climate regions.[5-7] As *A. esculentus* riches with a wide range of nutritional properties and provide potential health advantages, it is being used for medicinal and culinary purposes throughout history.[6,8] *A. esculentus* is also frequently used as ethnomedicine in a variety of cultures.[9] Its fruits are traditionally being used for cooling, stomachic, astringent, and aphrodisiac purposes.[10] In addition, *A. esculentus* also possesses powerful pharmacological activities such as antioxidant, neuroprotective, anti-inflammatory, antidiabetic, antihyperlipidemic and anti-fatigue effects.[7,8]

*A. esculentus* is primarily made up of water, carbohydrates, and proteins, only with a trace of fat and a moderate amount of dietary fibre. *A. esculentus* also contains potassium, sodium, magnesium, calcium, vitamins, folic acid, as well as iron, zinc and unsaturated fatty acids such as linolenic and oleic acids.[6,8,11,12] The phytochemical components in various segments of the *A. esculentus* plant are available in varying concentrations. According to previous study, the presence of phenolics, flavonoids, polysaccharides, vitamins, minerals and antioxidants in *A. esculentus* is responsible to have significant health benefits besides its biological activity.[5] The preliminary phytochemical screening also shows the existence of carbohydrates, gums and mucilages, proteins, phytosterols, flavonoids, tannins and phenolic compounds.[5,13] *A. esculentus* is known for its higher antioxidant properties due to the presence of a significant amount of phenolic compounds.[5,14,15]

According to a previous study, the phenolic and flavonoid content varies greatly across multiple *A. esculentus* cultivars gathered in Greece. The cultivars probably impact the antioxidant activity of those *A. esculentus*.[16] Besides, significant differences in phenolic compounds, antioxidant activities, and

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inhibitory effects on digestive enzymes were observed in different cultivars of *A. esculentus* fruits obtained in China.[17] In addition, qualitative and quantitative analysis of phenolic compounds of *A. esculentus* fruits from various cultivars are crucial and required for their biological features identification.[18,19] Consequently, to address the growing demand for high-quality vegetable crops with health-beneficial characteristics in Malaysia, it is vital to study the phenolic compounds and the bioactivities of *A. esculentus* fruits cultivated in Malaysia.

Although natural compounds are significantly beneficial, certain compounds gives harmful effects on human health.[20] The toxicological test is also required for complementary and alternative medicines apart from allopathic medicine, to detect the detrimental effects that are not apparent until the symptoms developed upon the excessive consumption.[21] Lower vertebrate and invertebrate organisms are commonly used as toxicity testing alternatives for higher vertebrates.[22] The zebrafish, scientifically known as *Danio rerio*, is a conventional animal model that is being used extensively in biomedical research to evaluate the toxicity of herbal plant bioactive components.[23] It has been reported that there is an 87% genetic similarity between zebrafish and humans, which tend to be associated with human disorders.[24,25] The embryo stages of zebrafish are preferred for toxicological evaluations due to the egg's transparency, which allows for direct observation of developmental phases and assessment of toxicity endpoints. These assays are cheaper, easy to handle with a shorter life cycle, greater fecundity, small amounts of test substances requirement, and high throughput screening making them a very ideal and successful model for toxicity studies.[25] Thus, the zebrafish embryo is an efficient assay for a wide range of toxicity studies as it provides rapid findings.[23]

Based on the previous studies, it has been proven that *A. esculentus* has a significant amount of polyphenol compounds. Therefore, this paper aims to evaluate the phytochemical constituents, and antioxidant activities of *A. esculentus* cultivated in Malaysia which has been extracted using multiple solvents. Besides, these extracts were also assessed for their toxic effects on the zebrafish embryo. The potential antioxidant properties with lower toxicity may assist in the mitigation of inflammation precursors that underline chronic inflammatory diseases.

## MATERIALS AND METHODS

### Sample collection and sample identification

The fresh *A. esculentus* samples were purchased from the local market at Semenyih, Selangor, which was harvested from a farm in Rawang, Selangor. The sample identity was authenticated by the plant taxonomist from Biodiversity Unit, Institute of Bioscience (IBS), Universiti Putra Malaysia. A voucher specimen (MFI 0248/22) was deposited in the IBS herbarium.

### Plant extract preparation

The fresh *A. esculentus* fruits were washed thoroughly with flowing tap water, dried at 50°C and ground to a fine powder. For each sample, 100 grams of ground fine powder were sequentially extracted with four solvents in the following order: (i) hexane, (ii) chloroform, (iii) methanol and (iv) aqueous. Initially, the extraction was carried out using hexane at a solid to solvent ratio of 1:10 (w/v) per extraction. The mixture was then left for 24 hours at room temperature. The hexane extract (HE) was separated from the mixture by filtration through a Buchner funnel with Whatman No.1 filter paper. The HE was then concentrated by using a rotary evaporator (Heidolph, Schwabach Germany) at 40°C under reduced pressure. The residue was air-dried and further extracted with chloroform (CE) and methanol (ME) similar to the procedure carried out for the hexane extract.[26] The extraction of aqueous was performed on the residue by adding distilled water at a solid to solvent ratio of 1:30 (w/v) per extraction. The mixture was then left for 24 hours at 2°C. The extract was coarsely filtered with 2 layers of gauge thrice, and centrifuged (Centrifuge 5810R) at 3500 rpm for 10 minutes.[27] The supernatant of aqueous extract (AE) was then freeze-dried by a freeze dryer (LSI Lyophilization System). The yield from all the extract was weighed and preserved at -20°C.

### Phytochemical screening

The presence of the phytochemical in the *A. esculentus* extracts was determined qualitatively using the appropriate established phytochemical methods.

### Anthraquinones

Borntrager's reaction: A 2 mL of each *A. esculentus* extract were added to 2 mL of 10% hydrochloric acid (HCl) and 2 mL of 10% of iron (III) chloride. It was then boiled for 10 minutes and filtered while hot. The filtrate was extracted with 5 mL of chloroform after completely cooled. The chloroform layer was tested with 2 mL of 10% ammonia solution. The formation of pink, red, or violet precipitate in the ammonia phase indicated the presence of anthraquinones.[28]

### Terpenoids

Salkowski test: A 5 mL of each *A. esculentus* extract was treated with 2 mL of chloroform followed by the addition of 3 mL of concentrated sulphuric acid ($H_2SO_4$). The formation of reddish-brown colouration at the interface confirmed the entity of terpenoids.[29]

### Flavonoids

Sodium hydroxide test: A 2 mL of each *A. esculentus* extract was treated with a few drops of 1% ammonia ($NH_3$). The formation of intense yellow colouration showed the presence of flavonoid compounds.[30]

### Saponins

Frothing test: A 5 mL of each *A. esculentus* extract was diluted with 5 mL of distilled water in a test tube. The test tube was shaken vigorously and was observed for the presence of stable persistent froth. Then, 3 drops of olive oil were mixed with the frothing formed and shaken vigorously again. The formation of an emulsion indicated the presence of saponin.[31]

### Tannins

Ferric chloride test: 0.5 mL of each *A. esculentus* extract was added with a few drops of 5% ferric chloride ($FeCl_3$). The presence of tannin was signified by an intense green, blue, or black colouration.[32]

### Phlobatannins

Hydrochloric acid test: A 1 mL of each *A. esculentus* extract was added with a few drops of 2% hydrochloric (HCl). The presence of phlobatannins was confirmed by the formation of a red-coloured precipitate.[29]

### Alkaloids

Dragendroff's test: A few drops of dragendoff reagent were added to 1 mL of each *A. esculentus* extract. Deposition of reddish-brown precipitate evidenced the alkaloid presence.[30]

### Cardiac glycosides

Keller-Killiani test: About 2 mL of each *A. esculentus* extract were added with 2 mL of glacial acetic acid, a few drops of 5% ferric chloride ($FeCl_3$) and 1 mL of concentrated sulphuric acid ($H_2SO_4$). Brown ring appearance at interphase confirmed the presence of cardiac glycosides.[29]

## Glycosides

Modified Bontrager's test: About 2 mL of each *A. esculentus* extract was treated with 3 mL of chloroform and 10% ammonia ($NH_3$) solution. The glycoside's presence was specified by the formation of rose-pink colour.[29]

## Reducing Sugars

Fehling's test: About 1 mL of each *A. esculentus* extract was diluted in 5 mL of distilled water and then filtered. Filtrates were heated after being added with a few drops of Fehling's A and Fehling's B solutions for a few minutes. The presence of reducing sugars was detected by the formation of a brick-red cuprous oxide precipitate.[33]

## Steroids

Salkowski's test: About 1 mL of each *A. esculentus* extract was treated with 1 mL of chloroform and a few drops of concentrated sulphuric acid ($H_2SO_4$). The formation of the brown ring indicates the existence of steroids, whereas a blue brown ring appearance signified the presence of phytosteroids.[29]

## Triterpenes

Salkowsli's test: About 1 mL of each *A. esculentus* extract was treated with 1 mL of chloroform and then filtered. The filtrates were shaken and left to stand after being treated with a few drops of concentrated sulphuric acid ($H_2SO_4$). The presence of triterpenes was confirmed by the golden yellow colouration.[34]

## Lipids

Stain test: A few drops of each *A. esculentus* extract were placed on the filter paper. The observation of an oil stain confirmed the presence of fixed oil.[35]

## Phenols

Ferric chloride test: About 1 mL of each *A. esculentus* extract was diluted into 2 mL of distilled water and treated with a few drops of 10% ferric chloride ($FeCl_3$). The presence of phenols was indicated by the deposition of blue or green precipitate.[29]

## Coumarins

Sodium hydroxide test: In 1 mL of each *A. esculentus* extract, 1 ml of 10% sodium hydroxide was added. The development of yellow colouration indicated the presence of coumarins.[29]

## Carbohydrates

Fehling's test: A portion of the *A. esculentus* extracts were diluted in 5ml of distilled water and filtered separately. Diluted hydrochloric acid (HCl), was used to hydrolysed the filtrates and neutralised with 10% sodium hydroxide and heated in a few drops of Fehling's A and B solutions. The presence of reducing sugars was indicated by the deposition of red precipitate.[34]

## Protein

Xanthoproteic test: A few drops of concentrated nitric acid were added to 1 mL of each *A. esculentus* extract. The presence of proteins was confirmed by the formation of a yellow colour.[34]

## Amino acids

Ninhydrin test: A few drops of 0.25% (w/v) ninhydrin reagent was added to 2 mL of each *A. esculentus* extract and boiled for 5 minutes. The presence of amino acids was indicated by the formation of blue colour.[29]

## Diterpenes

Copper acetate test: About 1 mL of each *A. esculentus* extract was treated with 3-4 drops of copper acetate solution. The presence of diterpenes was indicated by the formation of emerald green colouration.[36]

## Carotenoids

In a test tube, 1 mL of each *A. esculentus* extract was diluted with 10 ml of chloroform and vigorously shaken. The resultant mixture was filtered before being treated with 85% sulphuric acid ($H_2SO_4$). The presence of carotenoids was indicated by a blue colour at the interface.[37]

## Anthocyanins

About 2 mL of each *A. esculentus* extract was treated with 2 mL of 2 N hydrochloric acid (HCl). The existence of anthocyanins was indicated by the changes from pink-red colouration to purple-blue colouration when ammonia is added.[38]

## Betacyanins

Sodium hydroxide test: In 2 mL of each *A. esculentus* extract, 1 mL of 2N sodium hydroxide (NaOH) and boiled at 100°C for 5 minutes. The presence of anthocyanin is confirmed by a blue-green colour, while the presence of betacyanin was indicated by a yellow colour.[39]

## Total phenolic content (TPC)

The total phenolic content (TPC) of the *A. esculentus* extracts was determined by the Folin-Ciocalteu technique.[40] The 100 µL of standard gallic acid solution with the concentration range of 100-1000 µg/mL and 100 µL of each *A. esculentus* extract with the concentration of 1000 µg/L were pipetted into the centrifuge tubes. Thereafter, 4.5 mL distilled water, 100 µL of 2N Folin-ciocalteu reagent and 200 µl of 2% (w/v) of sodium carbonate solution were added to the tubes. The tubes were incubated for 3 hours in dark at room temperature. The aliquot of all samples and the standard solution were transferred to 96-well plates and the absorbance was measured at a wavelength of 760 nm using EnSpire® Multimode Plate Reader (PerkinElmer, USA). Triplicates were performed for each test sample. The TPC was determined from the gallic acid standard regression equation and was expressed as µg of gallic acid equivalent (GAE) per mg of the dry weight of the sample (µg GAE/ mg extract).

## Total flavonoid content (TFC)

The aluminium chloride colourimetric technique was performed to determine the total flavonoid content (TFC) of the *A. esculentus* extract according to the methods of Verawati et al., with some modifications.[41] The 0.5 mL of standard quercetin solution with the concentration range of 10-100 µg/mL and 0.5mL of each *A. esculentus* extract with the concentration of 1000 µg/mL, were pipetted into the centrifuge tubes, then mixed with 1.5 mL of ethanol. Subsequently, 0.1 mL of 10% aluminium chloride, 0.1 mL of 1 M sodium acetate and 2.8 mL of distilled water were added to the tubes. The tubes were allowed to stand at room temperature for 30 minutes. The absorbance of all samples and the standard solution was measured at a wavelength of 428 nm using EnSpire Multimode Plate Reader (PerkinElmer, USA). Triplicates were performed for each test sample. The TFC was determined from the quercetin standard regression equation and was expressed as µg of quercetin equivalent (QE) per mg of the dry weight of the sample (µg QE/ mg extract).

## Antioxidant activity

### 1,1-diphenyl-2-picrylhidrazyl

The 1,1-diphenyl-2-picrylhidrazyl (DPPH) radical scavenging activities of *A. esculentus* extracts were determined using the established DPPH

radical scavenging assay, based on the previous method with few modifications.[42] A 100 µL of the *A. esculentus* extract with a range of concentrations from 0 to 1000 µg/mL and the standard reference, ascorbic acid (AA) solution (0-100 µg/mL), were pipetted into a 96-microwell plate. The extracts were then added with 100 µL of 0.5 mM DPPH solution and were incubated at room temperature in dark for 45 minutes. The absorbance was measured at the wavelength of 517 nm using EnSpire® Multimode Plate Reader (PerkinElmer, USA). All of the test samples were performed in triplicate, whereas the data were presented as DPPH radical scavenging activity (%) and calculated using the equation as follows:

$$\% \text{ DPPH radical scavenging activity} = \{(A_{control} - A_{sample})/A_{control}\} \times 100\%$$

Where $A_{control}$ indicates the absorbance of the control, while $A_{sample}$ indicates the absorbance of the extracts/standard.

## Reducing power assay

The reducing power of *A. esculentus* extracts was obtained using the modified Fe (III) to Fe (II) reduction assay.[42] Briefly, 100 µL of the *A. esculentus* extracts and the standard reference, AA solution with a range of concentrations (0-1000 µg/mL) were pipetted into microcentrifuge tubes. Subsequently, 250 µL of 0.2 M phosphate buffer with a pH 6.6, 250 µL of 1% (w/v) potassium ferricyanide solution were added to the extract and then, the mixtures were incubated at 50°C for 20 minutes. Thereafter, 250 µL of 10% (w/v) trichloroacetic acid were added to a mixture before the centrifugation of the mixture at 3000 rpm for 10 minutes. Later, a 62.5 µL aliquot of the upper layer was pipetted into a 96-microwell plate and added with 62.5 µL of distilled water, followed by 12.5 µL of 0.1% (w/v) ferric chloride solution. The absorbance of the reaction mixture was measured at the wavelength of 700 nm using EnSpire® Multimode Plate Reader (PerkinElmer, USA). All of the test samples were carried out in triplicate, whereas the absorbance value per concentration of *A. esculentus* extract was used to express the reducing power. A higher absorbance value indicated the greater reducing power of *A. esculentus* extracts.

## Zebrafish embryotoxicity

### Zebrafish embryo

Zebrafish embryos from the egg spawn were utilised for each independent test. The 1-hour post-fertilisation (hpf) zebrafish eggs were supplied together with the Danio Assay Kit, which was purchased from the Biochemistry Department, Faculty of Biotechnology and Biomolecule Science, Universiti Putra Malaysia, Malaysia. The zebrafish embryo was incubated in Danio-SprintM Embryo Media containing 0.1% DMSO at 28°C.

## Preparation of test extract serial dilution

The two-fold serial dilution method was made to obtain a set of all *A. esculentus* extracts samples and positive control treatment, acetaminophen at multiple concentrations. The stock solution was prepared by diluting 10 mg of all *A. esculentus* extract and acetaminophen (APAP) in 1 mL of distilled water. A six series of concentrations were made from the stock solution: 7.81 µg/mL, 15.625 µg/mL, 31.25 µg/mL, 62.5 µg/mL, 125 µg/mL, 250 µg/mL and 500 µg/mL. The Danio-SprintM Embryo Media was used as a negative control.[21]

## Zebrafish embryo assay

Healthy embryos that were observed under the inverted microscope (Olympus CKX 41) at 6 hpf, were selected and transferred into each of the 96-well plates using a Pasteur pipette. Subsequently, the samples and control treatment were pipetted into each well in the 96-well plates based on multiple series of concentrations. The treated embryo development was monitored at 24, 48 and 72 hpf, to evaluate the embryo formation and development in each extract concentration. The survival rate, hatching rate, developmental defects in somites, tail detachment, otolith, blood circulation, heartbeat, malignant pigmentation and scoliosis are among the few parameters focused on the observation under the inverted microscope.[20,21]

## Data analysis

All the tests were done in triplicates and the results were presented as mean ± standard deviation (n=3). The lethal concentration at 50% ($LC_{50}$) of each *A. esculentus* extract and control treatment was determined, through the linear regression in the probit analysis using the mortality value against the log of test extract concentration.[21] The statistical significance of the multiple treatments was assessed using the one-way ANOVA test in SPSS 22.0.0 for Windows. The p-value of less than 0.05, which was attained from ANOVA analysis using Tukey's test specified that the results are statistically significant.

# RESULTS

The *A. esculentus* were extracted with different solvents; HE, CE, ME and AE. These extracts were utilised to determine the presence of phytochemical constituents, antioxidant capabilities and toxic effects of *A. esculentus*.

## Phytochemical screening

Table 1 below shows the various phytochemical constituents that were evaluated in different solvent extracts of *A. esculentus*. The presence of phytochemical constituents includes flavonoids, anthraquinones, saponins, tannins, alkaloids, reducing sugars, phenols and also diterpenes in all the extracts of *A. esculentus*. In contrast, phlobatannins and anthocyanins were absent in all the extracts of *A. esculentus*.

## Total phenolic content and total flavonoid content

Table 2 shows the TPC and TFC in 1000 µg/mL of *A. esculentus* extracts. CE and HE showed a significantly highest amount of phenolic content and total flavonoid content, respectively.

**Table 1: Preliminary phytochemical screening of *A. esculentus* extracts.**

| Phytochemical Constituents | HE | CE | ME | AE |
|---|---|---|---|---|
| Anthraquinones | + | + | +++ | ++ |
| Terpenoids | - | - | +++ | + |
| Flavonoids | +++ | + | +++ | ++ |
| Saponins | + | ++ | +++ | + |
| Tannins | ++ | +++ | +++ | +++ |
| Alkaloids | ++ | ++ | +++ | + |
| Cardiac glycosides | - | + | - | - |
| Glycosides | - | + | - | - |
| Reducing sugar | + | + | +++ | +++ |
| Steroids | - | - | ++ | - |
| Triterpenes | - | - | +++ | ++ |
| Lipids | + | - | +++ | - |
| Phenols | +++ | +++ | +++ | +++ |
| Coumarins | - | + | - | +++ |
| Carbohydrates | - | - | +++ | ++ |
| Protein | - | - | +++ | ++ |
| Amino acids | - | - | +++ | ++ |
| Diterpenes | + | ++ | +++ | ++ |
| Carotenoids | - | +++ | + | - |
| Betacyanins | - | - | ++ | +++ |
| Phlobatannins | - | - | - | - |
| Anthocyanins | - | - | - | - |

+++ = Present in abundant; ++ = Present in moderate; + =Present; - = Absent

## DPPH radical scavenging activity percentage of different extract of *A. esculentus*

As shown in table 3, the percentage of DPPH radical scavenging activity increased as the concentration of the extracts increased. The ME showed the highest scavenging activity (%) at 1000 µg/mL followed by CE, AE and HE.

## Reducing power of different extract of *A. esculentus*

Table 4 below shows the ferric reducing power of *A. esculentus* extracts and the standards, AA at multiple concentrations. The ME showed the highest ferric reducing power at 1000 µg/mL, followed by CE, AE and HE.

## Effect of different extract of *A. esculentus* on survival rate of zebrafish embryo

The survival rate of zebrafish embryos (in %) treated with different extracts of *A. esculentus* and APAP at multiple concentrations was observed at 72 hpf. The zebrafish embryo treated with HE, ME and AE showed a 100% survival rate for all concentrations ranging up to 500 µg/mL. The CE treatment was observed to demonstrate 33.33% and 0% of survival rates at the concentration of 250 µg/mL and 500 µg/mL, respectively. While, the standard treatment, APAP showed 100% of zebrafish embryo survival at all the concentrations.

## Effect of different extracts of *A. esculentus* on lethal concentration ($LC_{50}$) value on zebrafish embryo

The $LC_{50}$ value of zebrafish embryo treated with different extracts of *A. esculentus* and APAP were observed at 72 hpf. Table 5 summarised the finding for the embryotoxicity of the extracts. The positive control, APAP, did not show any $LC_{50}$ value.

## Effect of different extract of *A. esculentus* on hatching rate of zebrafish embryo

The hatching rate of zebrafish embryos treated with HE, ME and AE was 100% with ranging concentrations from 0.00 µg/mL – to 500 µg/mL. The hatching rate after 15.63 µg/mL to 125 µg/mL CE treatment was 100%. However, the hatching rate was 83.33% and 33.33% at the CE concentration of 7.81 µg/mL and 250 µg/mL, respectively. There was no hatching observed at 500 µg/mL of CE of *A. esculentus* treated zebrafish embryo. While 100% of hatching were observed on zebrafish embryo treated with APAP at multiple concentration.

## Effect of different extracts of *A. esculentus* on heartbeat of zebrafish embryo

The heartbeat (in beats per minute, bpm) of zebrafish embryo treated with multiple concentrations of *A. esculentus* extracts and positive control, APAP was shown in figure 1. All extracts; but ME and CE,

**Table 2: The TPC and TFC in 1000 µg/mL different extracts of *A. esculentus* (mean ± SD).**

| Extract of A. esculentus | TPC µg (GAE)/mg extract | TFC µg (QE)/mg extract |
|---|---|---|
| HE | 106.563 ± 0.442[a] | 63.757 ± 1.547[a] |
| CE | 143.854 ± 2.210[b] | 31.302 ± 0.170[b] |
| ME | 127.882 ± 2.385[c] | 36.765 ± 0.499[c] |
| AE | 121.076 ± 2.048[d] | 33.438 ± 0.299[d] |

[a, b, c, d]: p<0.05 as compared between type of extract.

**Table 3: DPPH radical scavenging activity % of different extracts of *A. esculentus* at multiple concentrations (mean ± SD).**

| Extract of A. esculentus | DPPH Radical Scavenging Activity (%) | | | | |
|---|---|---|---|---|---|
| | 200 µg/mL | 400 µg/mL | 600 µg/mL | 800 µg/mL | 1000 µg/mL |
| HE | 3.110 ± 0.454[a] | 6.256 ± 0.625[b] | 10.159 ± 0.688[c] | 11.192 ± 0.718[d] | 17.309 ± 0.547[e] |
| CE | 6.185 ± 0.104[a] | 10.884 ± 0.406[b] | 16.134 ± 1.187[c] | 18.540 ± 0.326[d] | 26.675 ± 0.111[e] |
| ME | 5.216 ± 0.551[a] | 10.126 ± 0.362[b] | 15.181 ± 0.244[c] | 20.720 ± 1.603[d] | 28.858 ± 1.806[e] |
| AE | 2.285 ± 0.292[a] | 4.652 ± 0.576[b] | 5.884 ± 0.300[c] | 8.795 ± 0.060[d] | 17.467 ± 0.363[e] |
| AA | 51.729 ± 8.139[a] | 90.622 ± 2.372[b] | 94.027 ± 0.122[c] | 94.104 ± 0.125[d] | 94.281 ± 0.115[e] |

[a, b, c, d, e]: p<0.05 as compared between the type of extract and AA at the same concentration.

**Table 4: Ferric reducing power of different extracts of *A. esculentus* at multiple concentrations (mean ± SD).**

| Extract of A. esculentus | Absorbance at 700 nm | | | | |
|---|---|---|---|---|---|
| | 200 µg/mL | 400 µg/mL | 600 µg/mL | 800 µg/mL | 1000 µg/mL |
| HE | 0.125 ± 0.001[a] | 0.133 ± 0.001[b] | 0.139 ± 0.001[c] | 0.146 ± 0.001[d] | 0.155 ± 0.001[e] |
| CE | 0.142 ± 0.001[a] | 0.161 ± 0.002[b] | 0.174 ± 0.001[c] | 0.198 ± 0.005[d] | 0.220 ± 0.001[e] |
| ME | 0.161 ± 0.001[a] | 0.184 ± 0.002[b] | 0.196 ± 0.001[c] | 0.233 ± 0.001[d] | 0.271 ± 0.00[2]e |
| AE | 0.152 ± 0.001[a] | 0.163 ± 0.002[b] | 0.175 ± 0.001[c] | 0.189 ± 0.002[d] | 0.217 ± 0.001[e] |
| AA | 0.189 ± 0.002[a] | 0.250 ± 0.001[b] | 0.290 ± 0.003[c] | 0.330 ± 0.002[d] | 0.370 ± 0.003[e] |

[a, b, c, d, e]: p<0.05 as compared between the type of extract and AA at the same concentration.

**Table 5: The lethal concentration at 50% of survival of zebrafish embryo treated with *A. esculentus* extract.**

| Extract of A. esculentus | $LC_{50}$ Value on Zebrafish Embryo |
|---|---|
| HE | No $LC_{50}$ at the maximum concentration of 500 µg/mL |
| CE | 236.07 µg/mL |
| ME | No $LC_{50}$ at the maximum concentration of 500 µg/mL |
| AE | No $LC_{50}$ at the maximum concentration of 500 µg/mL |

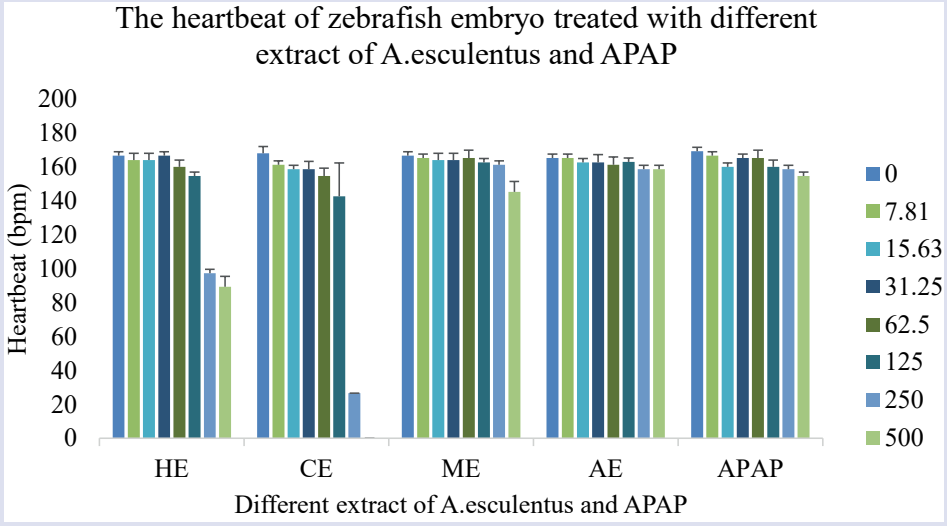


**Figure 1**: The heartbeat (in beats per minute, bpm) of a zebrafish embryo treated with different extracts of *A.esculentus* and APAP at 72 hpf. * is p<0.05 as compared to APAP.

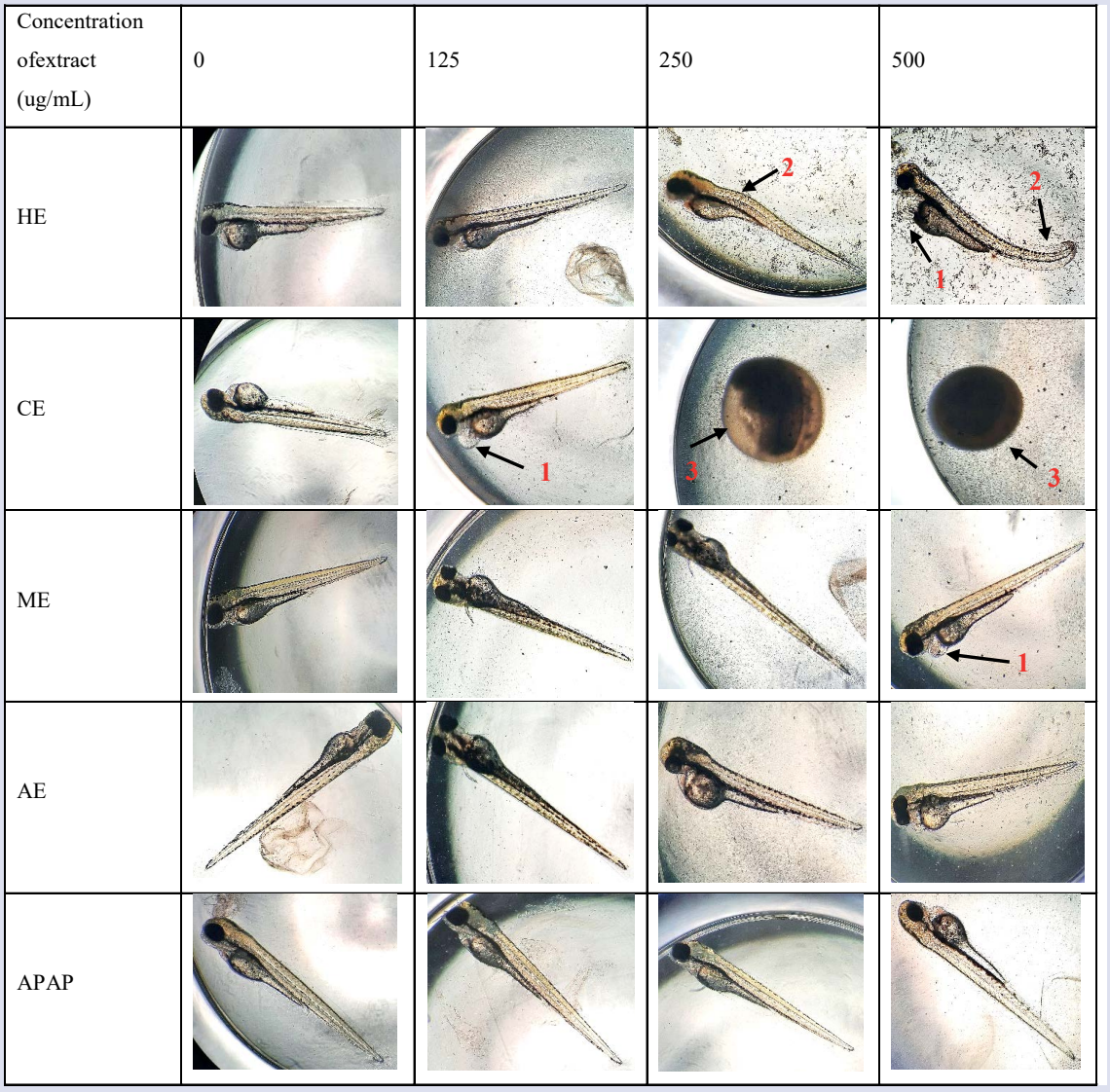


**Figure 2:** The abnormal morphological characteristics of zebrafish embryo treated with multiple concentrations of different extracts and APAP at 72 hpf observed under 5x magnification. Arrows indicate (1) pericardial oedema, (2) scoliosis and (3) coagulation/dead.

at all doses showed comparable Heartbeat as compared to APAP. The heartbeat of the embryo was recorded to decrease significantly after being treated with 250 and 500 µg/mL of HE and 500 µg/mL of CE. Figure 1 summarises the findings of the heartbeat of the embryo.

### Effect of different extracts of *A. esculentus* on morphological characteristics of zebrafish embryo

The morphological characteristics of zebrafish embryos treated with multiple concentrations of different extracts of *A. esculentus* and APAP were assessed at 72 hpf. The healthy growth of embryo was observed treated with 0 to 61.5 µg/mL of all extracts and APAP. Figure 2 shows the abnormal morphology characteristic of the zebrafish including the death embryo after treated with higher concentration of HE, CE and ME, as compared to the healthy embryo treated with AE and APAP.

## DISCUSSION

Besides its nutritional value, *A. esculentus* is being utilised for its therapeutics and nutraceutical qualities, due to the availability of multiple numbers of bioactive chemicals and related bioactivities.[43] The presence of phytochemical components known to have pharmacological and physiological properties can be discovered through phytochemical screening. The screening of secondary metabolites and other biochemicals inherent in the plant is significantly assisted by preliminary analysis.[44] Table 1 has summarised several phytochemical contents present in different extracts of *A. esculentus,* such as flavonoids, tannins, alkaloids and also phenols. The higher quantities of phytochemical contents were presented in ME compared to HE, CE, and AE of *A. esculentus*. These findings were supported by the reports from previous studies, which revealed the presence of alkaloids, flavonoids, carbohydrates, phenols, proteins, tannins, terpenoids, and sterols in AE and ME of *A. esculentus*.[45,46] In addition, carbohydrates, proteins, phytosterols, gums and mucilages, flavonoids, phenolic compounds and tannins were also observed in the phytochemical screening of cold AE of *A. esculentus* mucilage.[6,13] This discovery suggests that *A. esculentus* could be a novel phytopharmaceutical agent.

Polyphenols are secondary metabolic products of plants, that include phenolic acids, flavonoids, lignans, and stilbenes, and are present abundantly in food and medicinal plant parts.[47,48] The polyphenols serve as a free radical scavenger, inhibiting the formation of free radicals by adhering to metallic ions and altering the free radical chain's progression sequences.[49] Specifically, the most significant bioactive component discovered in *A. esculentus* fruit is the phenolic compound.[50,51] The phenolic compounds have antioxidant action due to their redox characteristics.[52] The CE of *A. esculentus* showed the highest phenolic content, followed by ME, AE and HE. The reports on antioxidant properties of *A. esculentus* are found to be inconsistent. The present findings contradicted the report by Ahmed & Sekar, revealed the highest TPC observed in ME, followed by ethanol extract and AE.[53] According to Oloyede & Ojeyinka, higher antioxidant properties was observed in HE as compared to ME.[54]

Besides phenolic content, the flavonoid content also was evaluated in this study. Flavonoids comprise hydroxyl groups that aid in the scavenging of free radicals and the chelation of metal ions, thereby modulating potent antioxidant activity.[55,56] Table 2 showed that HE of *A. esculentus* has the highest TFC, followed by ME, AE and CE. Similar to TPC, reports on the findings were found to be inconsistent. According to the findings from Ahmed & Sekar, it was revealed that ME was observed to have higher TFC compared to ethanol extract and AE of *A. esculentus*.[53] Interestingly, the TFC of five different cultivars of *A. esculentus* obtained in China were found to be varying.[17]

The TPC and TFC of *A. esculentus* extracts in this study were significantly diverged compared to the previous studies. Previous reports revealed that ME showed the highest TPC and TFC as compared to other extracts. The antioxidant potentials of the extracts discovered are entirely dependent on the solvents selected. This is due to the polarity of the bioactive chemicals, which results in varying rates of antioxidant properties.[57] As a consequence of the presence of various bioactive compounds in plants and the varying solubility qualities in different solvents, the best solvent to extract the compounds is determined by the particular plant derivatives and the compounds to be extracted themselves.[58] Besides, the extrinsic and intrinsic factors, such as cultivar, maturity and environmental circumstances, have a direct impact on plant phenolic profiles.[59] The interface of these factors would affect plant metabolism, resulting in the production of various bioactive chemicals, such as various types of phenolic compounds.[60]

The DPPH test is a commonly used method for determining the radical scavenging ability of plant extracts.[52] The present study showed the significantly increased scavenging activity of *A. esculentus* extracts, dose-dependently. The report revealed that ME possess the highest scavenging activity as compared to the other extracts, suggested to be influenced by the capability of methanol in dissolving primary active components, such as quercetin, catechin and vitamin C. The present data were consistent with the previous study that revealing the higher scavenging activities observed in ME as compared to AE. The amount of antioxidants found in these extracts is responsible to determine the DPPH radical scavenging activity.[26]

Another important measure of antioxidant activity is the extract's reducing capacity. The presence of antioxidants in the sample would enable $Fe^{3+}$ to be reduced to $Fe^{2+}$ by donating an electron in the reducing power assay.[61] The ME of *A. esculentus* showed a higher reducing power at the highest concentration similar to the DPPH scavenging activity. The reducing power among the extracts of *A. esculentus* increased as the concentration of dose increased. According to Doreddula et al., ME of *A. esculentus* showed a higher reducing power compared to the AE and the standard, butylated hydroxytoluene (BHT).[45] This is because the capacity of the methanol extract to reduce $Fe^{3+}$ to $Fe^{2+}$ is significant.[62] Several *in-vitro* studies have proven that the variation in phenolic contents may be the primary contributor to variation in the antioxidant capabilities of plants.[17,49]

Due to recorded incidences of adverse drug interactions, mutagenic, carcinogenic, and teratogenic effects, toxicological assessment of herbal medicine is becoming a major concern.[25] Zebrafish has recently gained popularity as a promising animal model for *in-vivo* high-content drug screening and toxicological evaluation.[63] Moreover, the European Centre for the Validation of Alternative Methods (ECVAM) has recognised zebrafishes as an excellent alternative animal model since the predicted success rate of zebrafishes for drug-induced toxicity achieved 100%.[64] The zebrafish toxicity studies have been approved to be utilised in new drug development research by the Food and Drug Administration for safety and toxicity evaluation.[65] Significantly, entire zebrafish seems to be capable of tracking toxic chemicals/constituents that are unlikely to be present in, *in-vitro* cell culture techniques.[66] In the form of a multi-wave plate format, higher volume screening platforms are available for examining chemical effects on the development of the embryo by assessing malformations, mortality, and structural features over a range of concentrations.[65]

The zebrafish embryotoxicity of *A. esculentus* extracts was evaluated by emphasising the survival rate, hatching rate, heartbeat rate, and other malformation of the embryo at 24, 48 and 72 hpf. The survival rate of organisms is described by their ability to acclimatise to a given environment according to their optimal condition needed. The pH, temperature, chemistry, nitrogenous waste factor, and hardness of water are among the optimum factors that can affect the survival rate of zebrafish embryos.[67] The present study showed that all extracts of *A.*

*esculentus* promoted a 100% of survival rate except for CE. A higher concentration of CE was found to cause a low survival rate in the embryo. Various phytochemicals are extracted in solvents of varying polarity depending on their chemical composition, as no particular solvent can reliably extract all of the phytochemical and antioxidant components inherent in plant material.[68] According to Khan *et al*., it was suggested that the phytochemical constituents of *A. esculentus* are responsible for its significant physiological and biological activity.[69] The lowest survival rate observed after the treatment of CE was postulated to influence by its lowest TFC as compared to other extracts. The flavonoids deficiency may cause oxidative stress to cellular molecules, resulting in decreased zebrafish embryo survival rates. Furthermore, DNA damage can be caused by bioactive chemicals such as saponin mixtures and alkaloids.[70]

To determine the safe concentration threshold for intake of different extracts of *A. esculentus,* $LC_{50}$ was calculated using the mortality rate of the zebrafish embryo. Generally, greater $LC_{50}$ values imply a low toxicity level because a higher concentration is required to achieve a 50% mortality rate in an organism.[67] The present study revealed that only CE of *A. esculentus* expressed the $LC_{50}$ value. The findings were consistent with the report by Sumana Roy *et al*., revealing the usage of chloroform as extract solvent presented with a high $LC_{50}$ value. These are relatable as the chloroform extract may cause toxic effects at higher concentrations.[71]

The external fertilisation occurs in the zebrafish embryo, and the eggs are deposited from the zebrafish yolk sack during the spawning stage. Later, the nonadherent embryo will ordinarily hatch around 48 to 72 hpf. The hatching rate of zebrafish is a significant element in its growth and development.[72] The normal hatching rate in zebrafish embryos can be assisted by the interaction of both the low choriolytic enzyme and high choriolytic enzyme to digest the chorion without any disruption from chemical substances.[73] The present study revealed that treatment of all extracts of *A. esculentus*, except for CE, promoted a 100% of hatching rate, comparable to APAP. The hatching rate of embryos treated with CE declined at a higher concentration, postulated to lead by the available chemical compound that caused the disrupts the behavioural and physiological functions. Hence, this signifies that the zebrafish embryo hatching rate of CE is dose-dependent.[74]

Heart rate variability is one of the most important cardiac function metrics that can be examined in embryonic zebrafish,[75] that provides information on the impact of chemical exposure on the cardiac regulating system.[76] Generally, the heartbeat of a growing wild-type zebrafish embryo begins at 36 hpf ranges from 120 to 180 bpm.[77] The heart rate of the embryo however can be disturbed by the chemical factors and temperature,[75] presented with bradycardia or tachycardia.[21] Figure 2 has summarised the heartbeat of the embryo after being treated with a different type of extract. According to Li *et al*., changes in a heartbeat were commonly influenced by toxicant exposure.[78] The most common impact of the toxicant exposure on the zebrafish embryo is the changes in the heartbeat.

The adverse environment might put the embryo under a lot of stress, impairing its growth and development stage and leading to morphological abnormalities.[79] Figure 3 shows the significant abnormal morphological characteristics of the embryo after treatment. Stress factors such as chemical compounds and heavy metals impact the morphological form of the teratogenic effect.[80] The existence of scoliosis infers that the treated samples are potentially toxic.[21] Exposing the zebrafish embryo to toxic compounds triggers it to develop pericardial oedema, circulating disturbance[81] and fluctuated heartbeat.[70] Treatment of CE causes coagulated embryos as that abnormal characteristic showed the lethal effect. The coagulated embryo is known as the core endpoint of an acute endpoint of acute lethality in zebrafish embryo development and growth.[82,83]

## CONCLUSION

The presence of key phytochemicals such as anthraquinones, flavonoids, phenols, saponins, tannins, alkaloids, reducing sugars and diterpenes were identified in HE, CE, ME and AE of *A. esculentus*. These different extracts of *A. esculentus* showed remarkable variation in TPC and TFC. Both the DPPH and reducing power assay exhibited varying degrees of antioxidant activities among the *A. esculentus* extracts. In addition, the toxicity findings suggest that AE of *A. esculentus* can be the best extract as it did not exhibit any toxic effect on zebrafish embryos including mortality and malformation. However, a detailed comprehensive experimental study should be performed to identify the specific compound of *A. esculentus* that exhibits a toxic effect on organs despite embryos. Thus, the phytochemical compounds and the rich antioxidant capabilities of *A. esculentus* extracts, may stimulate free radical scavenging activities at a least toxic level to control and reduce the precursors of inflammation which will result in chronic inflammatory diseases.

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## CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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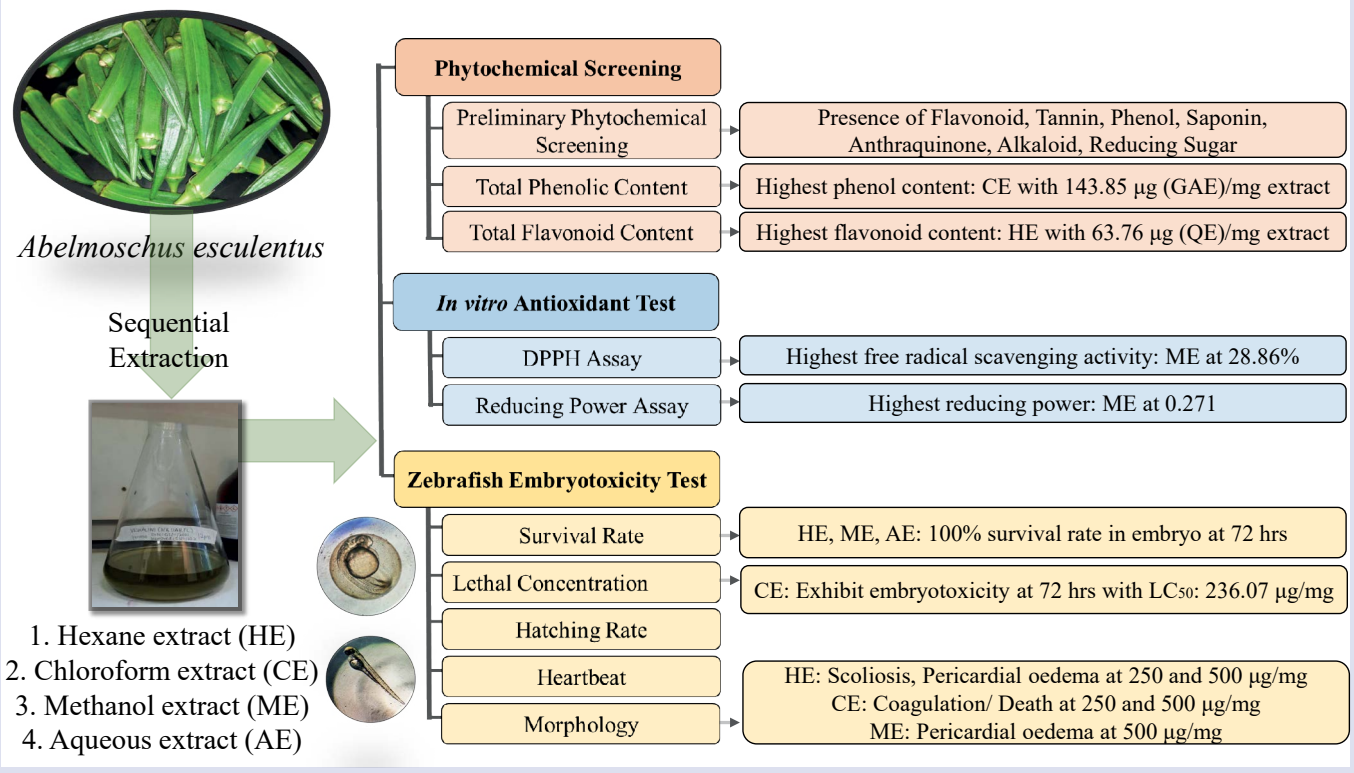
## GRAPHICAL ABSTRACT



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