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Research Article

Anti-putrefying properties of the aqueous extract of fresh leaves of *Manihot esculenta* (cassava) on dead laboratory Sprague Dawley rats

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Abstract
The leaves of *Manihot esculenta* (ME) have been used by ancestors, apart from food, to preserve human bodies before burial. Formalin remains the chemical agent of choice in modern times to preserve corpses. However, cost, associated health hazards and recent public health concerns meant that researchers must explore alternative means of continuing this age-long process of preservation of human remains. The present study aimed to explore the effectiveness of extracts from cassava *(Manihot esculenta)* leaves in the preservation of dead remains of corpses. Aqueous extracts from fresh leaves of *M. esculenta* (MELE) were used to preserve euthanised rats and the internal organs were harvested after 7 days for histological analysis. The histological sections of the stomach and liver were compared with those of control rats treated with 6 ml of formalin (10%). The low and intermediate doses of MELE preserved the tissues of the dead rats beyond 7 days, as evidenced by histological sections of the stomach and liver. Concentrations of MELE between 300 mg/kg and 1500 mg/kg showed adequate potency in preserving dead rats compared to formalin. However, the dead rats treated with doses of MELE greater than 1500 mg/kg showed rapid putrefaction after 7 days.

Keywords: Decomposition, Embalming, Putrefaction, Preservation, *Manihot esculenta*

INTRODUCTION

Embalming is the process of preserving a dead human body to delay decomposition for as long as possible (Brenner, 2014). Several reasons are ascribed to the embalming of the remains of a dead human, such as ranging from keeping a body for future generations to the necessity of having to transport a corpse from one country to another for burial or cremation and then the presentation of the body for public viewing (Ajileye *et al*., 2018; Brenner, 2014; Reddy *et al*., 2017) . Recently, formalin has become the chemical of choice for human cadaver embalming (Asare-Donkor *et al*., 2020). Denaturing of cellular proteins and subsequent inhibition of bacterial growth are thought to be the biochemical routes through which formalin has become an effective agent in preservation over the years (Brenner, 2014). However, it has also been reported to have toxic, allergenic and carcinogenic effects on those who frequently handle it (Asare-Donkor *et al*., 2020; Kawanishi *et al*., 2014).

Manihot esculenta (ME), commonly known as cassava plant in Ghana, is a woody shrub from the family *Euphorbiaceae*. It is an annual food crop in the form of a tuber, mostly cultivated in tropical and subtropical regions, as a great source of carbohydrates. The roots of the cassava plants serve mainly as food, which is very rich in starch and contains a significant amount of calci-

um, iron, manganese, phosphorus, potassium, dietary fibres, and vitamins B6 and C (Shigaki, 2016).The components and phytochemical analysis of *Manihot esculenta* leaf extract (MELE) have been well studied and documented in several scientific research (Chaiareekitwat *et al*., 2022; Chinnadurai *et al*., 2019; Clemen-Pascual *et al*., 2022; Rocha *et al.;* Shigaki, 2016; Tao *et al*., 2019).

Folklore has it that before modernization, the ancestors of some communities of the cassava-growing areas of West Africa used the leaves of this tuber as a form of embalmment aside from its use as food (Chaiareekitwat *et al*., 2022; Shigaki, 2016). A paste was said to be made from the leaves smeared on the corpses and burials delayed for a few days. These procedures were believed to 'strengthen' the corpse for reincarnation (Ajileye *et al*., 2018; Brenner, 2014), enhance the aesthetic look of the corpse in the afterworld (Ajileye *et al*., 2018; Dube *et al*., 2022) as well as remove any offensive smell and prevent the corpse from being destroyed by 'worms' (Brenner, 2014).

Indeed, recent studies on the extracts from the leaves of the plant were found to exhibit a broad-spectrum antibacterial activity, but no specific antibacterial agents were neither isolated nor identified (Lima *et al*., 2017; Mustarichie *et al*., 2020). The primary aim of this study was to determine whether MELE have any anti-putrefying properties on dead Sprague Dawley rats compared to an embalming agent such as 10% formalin.

MATERIALS AND METHODS

Collection of material

Leaves of cassava plants, *M. esculenta* (ME) were collected in April 2019, at a farm located at Kasoa, a town in the central region of Ghana. They were rid of soil and weighed. The leaves were air-dried for one week and milled into a powdered form, 300 g of the powder was then soaked in 3 litres of distilled water at 60°C for 6 hours and then filtered. The supernatant was collected and frozen at -80°C for 24 hours, freeze-dried for 48 hours (Zakaria *et al*., 2006) and the powdered sample was kept in a storage room under optimal conditions pending administration into the yet to be euthanized, animal models.

Sterility of MELE

Microbiological filtration and testing procedures were performed (Sachir *et al*., 2022) on the powdered substance by weighing 31.095 g of it and dissolving in one litre (1L) of distilled water to make a concentration of 31.095 g/L. One (1) litre of the sample was filtered through cotton wool and then subsequently through a Whatman filter paper. A 0.8 um filter was thereafter used to filter this sample using a suction, followed by a 0.2 um filter. All these procedures were undertaken in a sterilized biosafety hood.

The filtrate was subjected to a sterility test using a 3.5g plate count agar, which was weighed accurately and dissolved using 200 ml of hot distilled water (Ṣachir *et al*., 2022; Shami *et al*., 2013). The resulting solution was kept in a water bath at 100^{O}C to allow good mixing and then autoclaved at 120^{O}C for 15 minutes.

A thioglycollate broth medium was later prepared by weighing 1g of thioglycollate powder and dissolving in 200 ml of distilled water. The resulting solution was poured into different test tubes, plugged with a ball of cotton wool, and autoclaved at 120^{O}C for 15 minutes. The broth and agar were cooled and dilutions of the filtered sample were made in 10^2, 10^4 and 10^6. For the first dilution 10^2, 1 ml of the sample was taken and added to 99 ml of sterile water. The second dilution was done by taking 1 ml of the first dilution and adding 99 ml of distilled water. The third one was done by taking 1 ml of the second and adding on to 99 ml of sterile water. Finally, 1 ml was taken from each dilution and inoculated into the plate count agar using the pour plate method. Also, 1 ml of each dilution was added to the thioglycolate broth. The resulting media was cultured at 37^{O}C for 48 hours. After 48 hours, there was no growth in all the plates. Thus the extract was regarded as sterile.

Animal husbandry

Fifteen 2 to 3 months old male Sprague-Dawley rats weighing between 110 and 180 grammes were used in this study. They were fed with certified animal feed with very low contaminants for 14 days. This was obtained from Kosher Feed Mills Ltd, Osu, Accra. Distilled water was given *ad libitum* before acclimatisation as described (N'guessan *et al*., 2022). Feed was withdrawn 8 hours prior to treatment to ensure adequate absorption from the GIT after oral administration (Asiedu-Gyekye *et al*., 2014; N'guessan *et al*., 2022).

Animal groupings

All the rats were divided into five groups of three before being euthanized using chloroform soaked in cotton wool to put the animals to sleep and then subsequently euthanized. The dead rats were fixed with adequate doses of the extract and formalin (for the controls) through the rectal, intraperitoneal and oral routes to serve as an embalming fluid. The dosing and groupings were as follows:

Group I - high dose of the MELE - 3000 mg/kg
Group II - intermediate dose of 1500 mg/kg MELE
Group III - low dose of 300 mg/kg of MELE
Group IV - negative controls. No MELE and no formalin solution
Group V - positive control, 6 ml of 10% formalin solution

All the dead rats were kept at room temperature for two weeks. The stomach, liver and kidney from these treated dead rats were harvested on day 7 and day 14 after treatment and processed for routine light microscopy histological tissue processing analysis. Hematoxylin and eosin staining procedure (Ramaswamy and Dayasagar, 2017) was adopted in staining harvested tissues for microscopy. A Leica 400 light microscope was used to view the slides for analysis.

Ethical approval

The care and use of animals were in full compliance with national and international laws and guidelines on using experimental animals for research (Demers *et al*., 2006; Mandal & Parija, 2013). The study was approved by the Animal Research and Protocol Review Committee of the College of Health Sciences of the University of Ghana with protocol number EPRC/18/175.

RESULTS AND DISCUSSION

The stomach and liver of the rats were purposefully harvested for histological analysis since these organs are the first to undergo decomposition upon death. It has been reported that during early decay, intrinsic bacteria begin to digest the intestines starting from the epithelial lining through to the outer coat and the surrounding accessory organs (Hyde *et al*., 2013; Roy *et al*., 2021). By gross inspection, all the embalmed rats were well preserved after 7 days, which is what the embalming industry aims to achieve – aesthetic cosmetic preservation rather than histological preservation (Ajileye *et al*., 2018; Dromaguet *et al*.; Reddy *et al*., 2017). Therefore, the effect of the MELE (cassava leaf extract) could be compared to some extent with orthodox embalming agents. This preservative potential of the extract may be due to its composition, as documented. The effectiveness of the different doses of MELE on retarding putrefaction was evidenced by the histopathological analysis of some selected organs (Fig. 2 and 4).

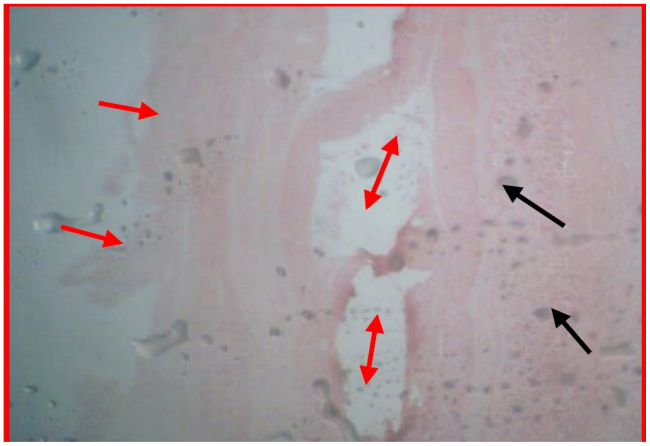
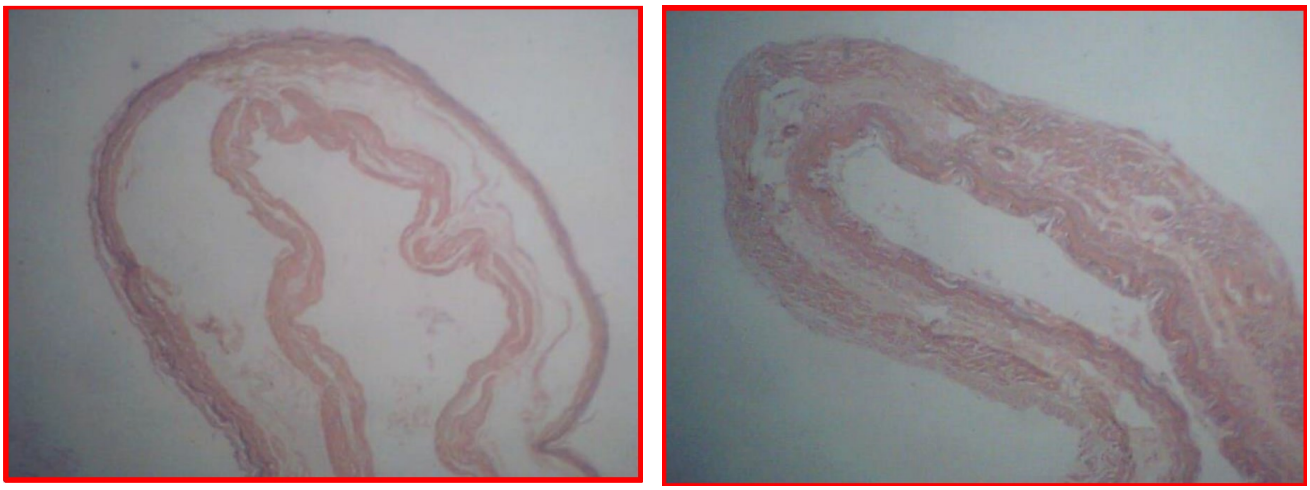


Fig. 1. *Section of stomach of negative control showing advanced staged of decomposition after 7 days of storage: Note the faintly denuded epithelial lining red arrows (left); Muscular coat of the stomach characterised by large cavities (double-headed arrows); Tiny dark spots (black arrows) scattered throughout the slide from the submucosal lining through to the muscular layer (black arrows), likely to be rapidly decomposing tissues) (Low power magnification (x4).*

The negative controls showed visible morphological decomposition by day 7 of treatment (Fig. 1). Compared with the positive controls and the extracts, the mucosal lining of the stomach, the hepatocytes and the connective tissue network of the liver showed signs of decomposition with slides of tissues obtained from the negative controls (neither embalmed with formalin nor



A **B**

Fig. 2. *Section of stomach of positive control i.e.* ***A.*** *formalin embalmed and* ***B.*** *low dose MELE embalmed rat tissues) showing the entire preserved histology. Note the lumen with the underlying mucosal lining through to the serosa, all histological layers of the stomach appear distinct and preserved in both slides (lower power magnification (x4)*

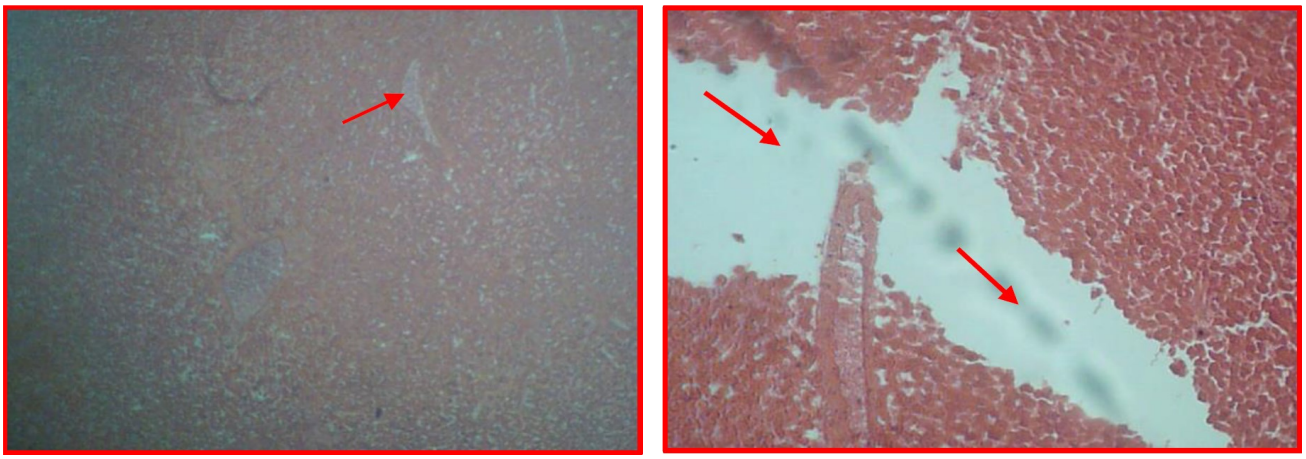
the extracts). This was in sharp contrast to the positive control (formalin-embalmed dead rats) as well as the low and intermediate doses of MELE, in which the mucosal lining and some subcellular components, as well as organelles in the liver, appeared to remain intact, thus thought to be preserved even at day 7 after death. Decomposition of the liver tissues appears to be radiating from the central vein (arrowed) into the adjoining connective tissues, as seen in the slides. These slides revealed large portions of the liver with pyknotic appearances (Fig. 2 and Fig. 6).

The efficacy and existence of microbicidal activity of the extracts of most plants belonging to the Euphorbiaceae family because of their peculiar flavonoid content have been reported (Kirbag *et al*., 2013; Pisano *et al*., 2016; Waheed *et al*., 2020). However, to the best of our knowledge, this is the first study to link these pharmacological properties to anti-putrefaction. Hence the embalming of dead animal remains. To achieve this, novel methodologies were used to ascertain whether the results of treating rats with MELE would be comparable to the routine embalming agents. The findings of this study have to a large extent, demonstrated that MELE could yield optimal/comparable results when used in moderation. The intent of embalming is not to prevent autolysis completely but to halt or retard the decomposition rate to an appreciable level (Ajileye *et al*., 2018).

To a considerable extent, the optimally formulated concentration of this extract can achieve this objective, as shown in the low to moderate doses of MELE in this study. Fig. 5 and Fig. 6, however, showed the extent to which high doses of MELE facilitated the decomposition rate much the same way as the negative controls. In these cases, the present study observed the extent to which the integrity of the stomach and liver was highly compromised. The study also tested the pharmacological and pharmacokinetic dynamics of the extract against the histological integrity of those organs/tissues of the rat obtained from the most likely systems prone to early autolysis– the gastrointestinal tract and its accessory organs. These results suggest that MELE could be harnessed and probably serve as a potential alternative to the current embalming agent in the mortuary industry since this plant extract is known to be environmentally friendly (Brenner, 2014). There have been reports of possible health hazards, including carcinogenic effects associated with the prolonged use of formalin in the embalming industry (Asare-Donkor *et al*., 2020). The expectation, therefore, is that this study would trigger the curiosity of scientists and researchers to research further on the possible efficacies of this plant extract to harness its potential benefits.

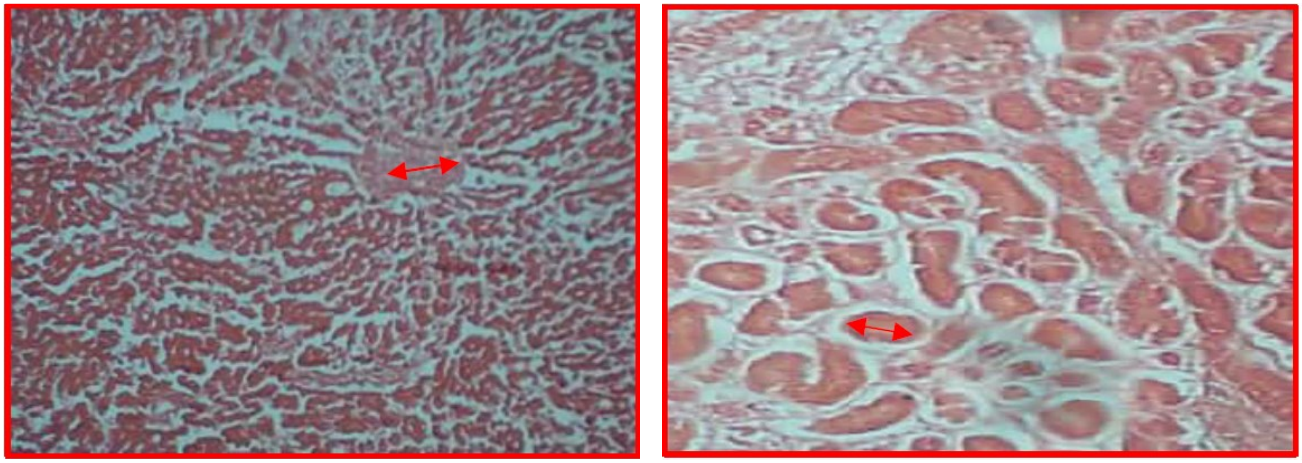
It is stated that the histological slides were prepared to support the pharmacological and anti-putrefying activities of the extract and, more especially, for the potential to mimic as much as possible for the micro-anatomical appearance of tissues of dead remains. Additionally, using the histological slides in this study is meant to strengthen our observations and demonstrate the histopathological way the proteins are denatured and how various organs and tissues remain fixated to the embalming industry. This is by no means an attempt to depict histological tissue processing and preservation of organs for analysis of finer details. It has been reported that in the traditional embalming industry (mortuary), the emphasis is on retarding the process of autolysis for a short period but never on histological tissue preparation, of which techniques and processes of obtaining the dead remains, typically differ from the method right from the onset (Ajileye *et al*., 2018). In the embalming industry, where committal, burials and incinerations usually follow the short period of display (laying-in-state) of a supposed fixed body, hence there is virtually no regard for strict preservation of detailed subcellular contents (Brenner, 2014) in the embalming industry. In the current study, the extract can fairly match this retardation of decomposition activity in comparison to the positive controls (formalin). From a comparative point of view, the use of histological analysis was to focus on the epithelial lining of the digestive tract, which was usually laden with different types of flora and fauna that could, in turn, accelerate the process of decomposition, a few hours after death in the absence any anti-putrefying agent. Therefore, the observation that the epithelial lining remained intact in no other site of the gastrointestinal tract than the stomach, a major storage site of flora and fauna several days after death, is an affirmation of the potency of this extract in the retardation of the rate of putrefaction. It is not yet clear the mechanism by which the high concentrations of the extract failed to achieve this process of retardation of putrefaction but rather facilitated the rate of decomposition rapidly. However, evidence from methodology indicated that the anti-putrefying activities of the cassava leaf extract are dose-dependent, an observation that also requires further investigation. The high dose of the extract as depicted in Fig. 5 and 6 was comparable to the controls. The present study recommends a low dose for embalmment purposes. High flavonoid contents often serve as a medium of growth for flora and fauna lining the gastrointestinal tract (Tao *et al*., 2019). Thus in the high dose extract, the high flavonoid concentration rather may have turned to accelerate the decomposition rate in sharp contrast to the use of lower concentrations of the extract.

The histopathology of the liver was also processed to affirm the potency of MELE against putrefaction. This is an organ regarded as one of the accessory glands of the digestive system, with high rates of metabolic activities and serving as an interphase between the digestive and cardiovascular systems (Ajileye *et al*., 2018; Asare-



A B

Fig. 3. *Section of the liver of negative control showing showing autolytic activities on day 7 of death. Note the highly pyknotic nature of the slide on the left* ***(A)*** *and the presence of large cavities (right and arrowed)* ***(B)****, indicating advanced stages of decomposition (low power magnification (x4)).*

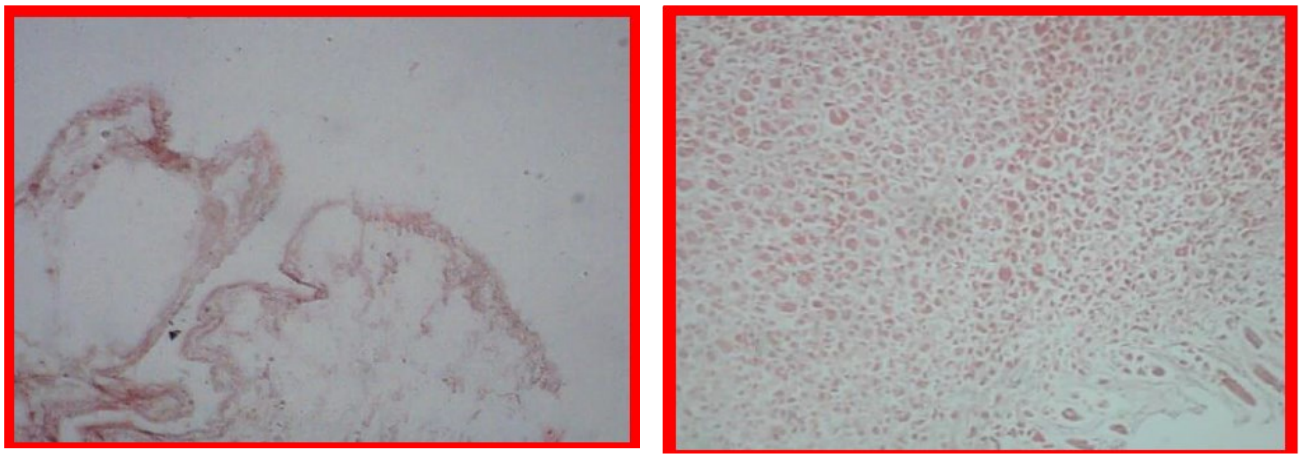


A B

Fig. 4. *Section of liver of positive control-****A*** *and low dose MELE-****B*** *at day 7 showing a comparable resemblance of the typical micro-architecture of a mammalian liver. Note the central vein of the liver (double-headed red arrows) around which are surrounded by tiny lobules, from both slides as well as the presence of traces of wide areas representing the liver reticular fibres (connective tissues). These strands of fibres are shown as white ramifications (low power magnifications (x4)*

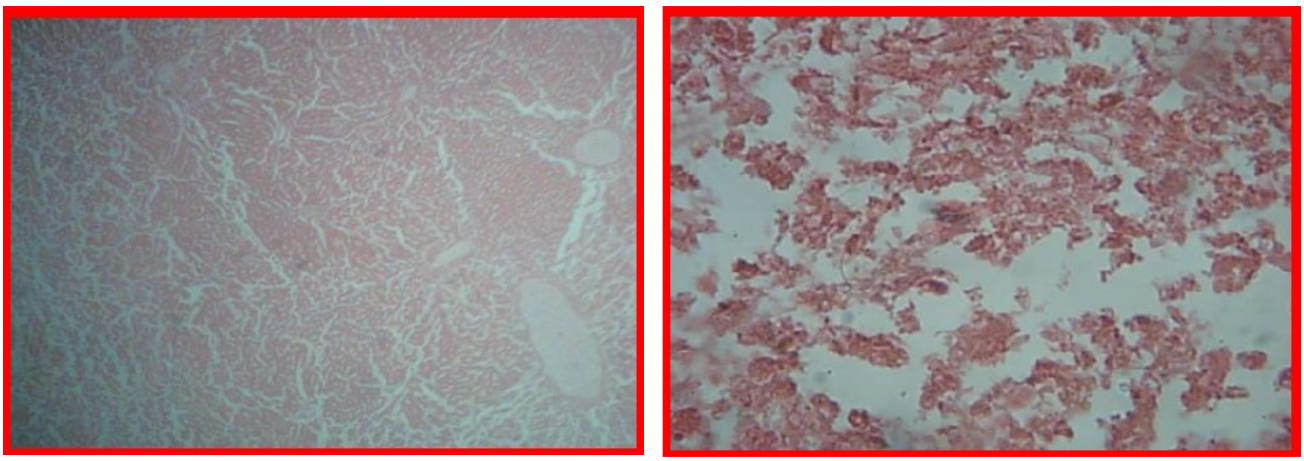
Donkor *et al*., 2020; Hyde *et al*., 2013). Therefore, the liver's histology findings could serve as a supportive piece of existential evidence for the study. The present study presumed that death could mitigate against the various biochemical activities occurring in the liver, thereby leading to sequelae of events and a probable onset of rapid decomposition in this organ moments after the cessation of breath. It was decided not to limit the present investigation of MELE on the epithelium of the gastrointestinal tract only, but also on the liver i. e. hepatocytes, Kupffer cells, endothelial cells and the fibres (connective tissue cells), which is more heterogeneous than that of the stomach and may provide an additional piece of evidence to buttress our hypothesis.

Indeed the present findings revealed that for the negative controls, the critical organs under review in this study (stomach and liver) have gone bad histologically despite being kept in a refrigerator. The entire mucosal lining of the stomach was degraded, with the epithelium completely non-existing (Fig. 1). Similarly, the submucosa and mucosal linings also revealed advanced stages of decomposition, as evidenced by numerous fluid-filled cavities. Slides of the liver from the negative control group could also be seen showing visible signs of active decomposition, with the central vein leading the way (Fig. 3). Comparatively, histological tissues obtained from the low-dose MELE-treated rats and those from the positive controls still revealed near-normal histological presentation at day 7 of death. Further, in Fig. 4, there was a characteristic representation of the histology of the liver of the rat. Their relatively pinkish to maroon colourations reveal the presence of preserved



A B

Fig. 5. *Section of stomach of rats embalmed with high dose of MELE extract at day 7 showing the compromised integrity of the epithelial and submucosal layers of the stomach.* ***A.*** *X4 magnification shows mucosal and submucosal layers at different stages of decomposition.* ***B.*** *X10 magnification focuses on the distortion seen in the cells (pyknotic) of the submucosal layer*



A B

Fig. 6. *Section of liver of rats embalmed with high dose of MELE extract at day 7 showing the highly pyknotic nature and presence of large cavities in the liver indicating advanced decomposition.* ***A.*** *X4 magnification shows advanced stages of decomposition, as shown by the numerous white spaces in the liver parenchyma.* ***B.*** *X10 magnification shows clearer signs of tissue distortion (white spaces) with no evidence of preservation of the classical liver lobule nor its and associated central vein.*

hepatocytes constituting large proportions of the liver parenchyma. There appears to be a differential discolouration between slides obtained from the formalin-embalmed liver tissues and that from the low-dose extract.

The pyknosis and karyorrhexis observed are by no means a negation of the potency of the anti-putrefying activities of the extract nor formalin but a normal pathological reaction of dead cells. Notably, the MELE low dose slowed down these activities comparable to the formalin-embalmed rats without progressing to active decomposition even at 7 days after. This slowing down and potential anti-putrefying activity of MELE might be possibly due to the components and phytoconstituents of the aqueous extract (Chaiareekitwat *et al*., 2022; Dube *et al*., 2022; Rocha *et al*.; Tao *et al*., 2019). Preserving the histology of the stomach with its epithelial lining and that of the liver with its central vein and surrounding connective tissues intact by the low dose of MELE presents substantial evidence requiring further investigation of the anti-putrefying potentials of *M. esculenta* plant. This could be a new molecule to be exploited in the embalming industry.

Conclusion

The present study concluded that the extract of *M. esculenta* leaves (MELE) when administered to the dead laboratory Sprague Dawley rats in low doses, the doses of between 300 mg/kg and 1500 mg/kg were capable of preserving tissues of the dead laboratory rats and preventing decomposition in comparison to forma-

lin preserved tissues. However, aqueous extracts of the leaves of greater than 3000 mg/kg facilitated the decomposition of the dead laboratory rats rapidly. This may probably be due to the high flavonoid content favouring microbial growth compared to the low doses. Further investigations into the anti-putrefying activities as well as the dose-related pharmacological and pharmacokinetic properties of the cassava leaf extract, are highly recommended.

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Conflict of interest
The authors declare that they have no conflict of interest.

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