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In-Vivo Acute Toxicity Test of Bioactive Phytochemicals In locally used Mango and Soursop Leaves using Laboratory Mice

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ABSTRACT: The consumption of plant-based herbal formulations have been on the increase in recent times, due to their medicinal properties, however, the identification, isolation and characterization of their bioactive constituents for specific health therapy and their safety have remained an issue of concern. This research identified, isolated and characterized succinic acid from saponin fraction of *Mangifera indica* (mango leaf SPEM) and hexamethyl cyclotrisiloxane from flavonoid fraction of *Annona muricata* (soursop leaf SPES). Cold maceration was used for extraction with ethanol as the solvent. GCMS of the samples mass spectra was analysed and gravimetric methods used for the extraction of the bioactive constituents. Results from screening of both leaf samples revealed the presence of alkaloids, flavonoids, tannins, phenols, terpenoids and saponins. Quantitative analysis of crude ethanolic extract yielded 15.15% and 20.60% respectively for mango leaf and soursop leaves. The selected phytochemicals for both samples yielded high for flavonoids and saponin, followed by phenol and tannin then alkaloids and terpenoids that gave the least yield. Succinic Acid and Hexamethyl Cyclotrisiloxane were selected respectively as the predominant organic compounds, isolated and administered in two phases to a group of randomly distributed mice for acute toxicity test (LD_{50}). The first phase involved the oral administration of 10, 100 and 1000 mg/kg body weight, and 1600, 2900 and 5000 mg/kg body weight of the extracts. Results showed that the saponin fractions of SPEM (Succinic Acid) were found to be non-toxic at doses ≤ 1000 mg/kg b.w (body weight) while the flavonoid fraction of SPES (Hexamethyl Cyclotrisiloxane) was non-toxic at doses ≤ 5000 mg/kg b.w. We conclude therefore, that both phytochemicals are safe for oral administration at low doses of about ≤ 1000 mg/kg b.w

KEYWORDS: Mango leaf, Soursop leaf, Acute toxicity, Phytochemical, Extract and Mice.

1. INTRODUCTION

Plants contain various phytochemicals that are of health importance (Samuel C. Iwuji, Sixtus A. Okafor and Chioma C. Okey-Mbata 2020). The use of herbal remedy alone or in addition to the conventional medicine has become common practice in the management of diseases around the world Agu, K. C., & Okolie, P. N. 2017), especially in developing countries including Nigeria. This is because herbal medicines are considered cheap and locally available (Verma *et al*., 2018). The availability of these medicinal plants is associated with climatic conditions that influence the growth and viability of the various species (Mahammed *et al*., 2015). Most of these plant species, though, are usually used as food or spices, however, their use for medicinal purposes, are still under developed, although, over the years, efforts to harness their potentials towards the formulation of more effective and safer drugs are on the increase (Okafor *et al*., 2021).

A number of these plants have the ability to reduce glucose production, induce glucose utilization, boost immune system, exhibit anti microbial potentials and combat many diseases and infections based on their phytochemical compositions. Some of these major bioactive components which include: Alkaloids, Flavonoids, Glycosides, Polysaccharides, Sterols, Peptidoglycans, Amino acids and their derivatives, Saponins, Terpenoids, Carotenoids are abundant in plants (Ardalani *et al*., 2021; Tran *et al*., 2020).

Mango and soursop leaves extract possess inherent potentials in the management of diseases including diabetes (Samuel C. Iwuji *et al*., 2021 and Sixtus *et al*., 2022). They have been implicated in the lowering of blood glyceamic levels (Okafor *et al*., 2022) and boosting of immune system; as they contain a variety of bioactive compounds such as phenol, terpenoids, sesquiterpenes, benzophenones, flavonoids, alkaloids, saponins, tannins and xanthones (Kumar *et al*., 2021). There is paucity of information on the safety of most of these herbal mixtures and their safe dosage for administration. This study, however, is carried out to determine the in-vivio acute toxicity of the bioactive phytochemicals in the ethanolic leave extract of the plants.

2. MATERIALS AND METHODS

2.1 Materials

Materials used include: Leaves of Annona muricata (Soursop leaf) and Mangifera indica (Mango leaf); Analytical grade chemicals and reagents purchased from registered chemical companies/ outlets. Alpha amylase (Molychem), Ethanol (Emsure), Methanol (Sigma-Alorich), Sodium Chloride (JHD), Petroleum Ether (JHD), Ammonium Hydroxide (JHD), n-butanol, n-hexane, Folin-Denis reagent, standard tannic acid, DPPH, Potassium ferrocyanide, H2SO4, Sodium phosphate, Ammonium molybdate, Starch azure, Sodium acetate buffer, Cacl2 and Trichloroacetic acid (TCA), Acetone, Ammonium sulphate (NH4)2SO4 (ECCL England), Activated charcoal and Acetic acid.

2.2 Specimen Preparation

The leaf samples were washed under tap water and air-dried at normal room temperature for 4 weeks, then pulverized into powder using an electric blender and stored in airtight ziplock samples bags to avoid moisture and contamination.

2.3 Extraction of Phytochemicals

Crude ethanolic extraction of the plants phytoconstituents was carried using 95% ethanol in a sample to solvent ratio of 1:6 (w/v) in an air-tight, well labeled specimen bottle. The content was left to stand at room temperature for 3days, with occasional agitation to ensure complete extraction, followed by filtration using muslin cloth folded into two. The extract was concentrated under vacuum in a rotary evaporator with the heating bath set at 45^0C.

2.4 Phytochemical Analysis

Phytochemical screening tests of the ethanolic extracts were carried out according to (Roghini & Vijayalakshmi, 2018), Alkaloids determined according to the method of Harborne (Roghini & Vijayalakshmi, 2018), Saponin content determined according to (Roghini & Vijayalakshmi, 2018), Terpenoid content determined according to (Azeemi, 2017), Flavonoid determined by the method reported by Boham and Kocipai in (Ezeonu & Ejikeme, 2016), Total phenolic contents determined according to the method described by (Phuyal *et al*., 2020) and Tannin content determined as described by Pearson (Bukola, Catherine & Gabriel, 2018).'

2.5 In-Vitro Acute Toxicity Assay

The LD_{50} of the selected phytochemical derivatives were conducted according to Lorke's method (Chinedu *et al*., 2013). Doses of the Mange leave extract and Soursop leave extract derivatives were administered in two phases. The first phase involved the use of nine (9) mice randomly distributed into three groups of three mice and administered 10, 100 and 1000 mg/kg body weight of the extract orally. The second phase involved 1600, 2900 and 5000 mg/kg body weight of the extracts. The mice were monitored for 24 hours for mortality and LD_{50}, calculated as the geometric mean of the maximum dose that caused no death and the minimum lethal dose.

3. RESULTS

The preliminary phytochemical screening test of crude ethanolic extracts of *Annona muricata* (Soursop leaf) labelled as SPES and *Mangifera indica* (Mango leaf) labelled as SPEM, revealed abundance of alkaloids, flavonoids, tannins, phenols, terpenoids, and saponins.

Table 3.1: Qualitative analysis of ethanolic extract of Mango leaf (SPEM) and soursop leaf (SPES).

S/N	Extract	Cho	Tan	Sap	Alk	Fla	Gly	Qui	Phe	Ter	Cg	Nin	Cou	Aq	Ste
1	SPES	++	+++	++	++	+++	-	+	+++	+++	-	-	++	-	++
2	SPEM	++	+++	+++	+++	+++	-	+	+++	+++	-	-	++	-	++

Ethanolic extract of powdered leaf samples of SPEM (Mango leaf) and SPES (Soursop leaf) by cold maceration yielded 15.51% and 20.60% respectively, which shows that soursop leaves produced a higher yield than Mango leaves.

Carbohydrates (Cho), Tannins (Tan), Saponins (Sap), Alkaloids (Alk), Flavonoids (Fla), Glycosides (Gly), Quinones (Qui), Phenols (Phe), Terpenoids (Ter), Cardiac glycosides (Cg), Ninhydrins (Nin), Coumarins (Cou), Anthraquinones (Aq) and Steroids (Ste)

(SPEM) = Mango leave extract

(SPES) = Soursop leave extract

(+) = indicates the yield of the various phytochemical

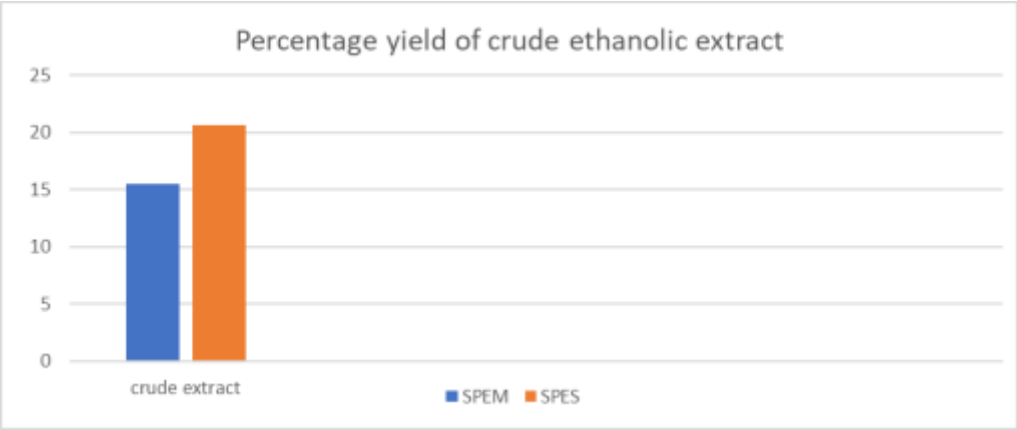

Figure 3.1. Percentage Yield of Crude Ethanolic Extract of SPEM (Mango leaf) and SPES (Soursop leaf) by cold maceration yielded 15.51% and 20.60% respectively.

Phytochemical content of SPEM and SPES was observed high for flavonoid (0.0502% / 0.0414%) and saponin (0.0471% / 0.0431%) followed by phenol (0.01742% /0.02473%) and tannin (0.0172% / 0.0164%). Alkaloid gave a low yield (0.0022% / 0.0019%) whereas terpenoid yield was not significant (0.0002% / 0.0003%) respectively.

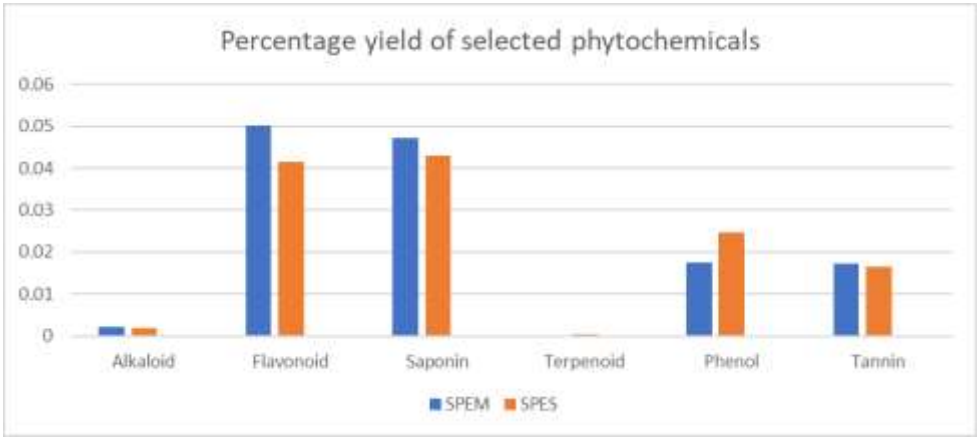

Figure 3.2. Percentage Yield of Selected Fractions of SPEM and SPES. Alkaloid (0.0022% / 0.0019%), Flavonoid (0.0502% / 0.0414%), Saponin (0.0471% / 0.0431%), Terpenoid (0.0002% / 0.0003%), Phenol (0.01742% /0.02473%) and Tannin (0.0172% / 0.0164%) respectively.

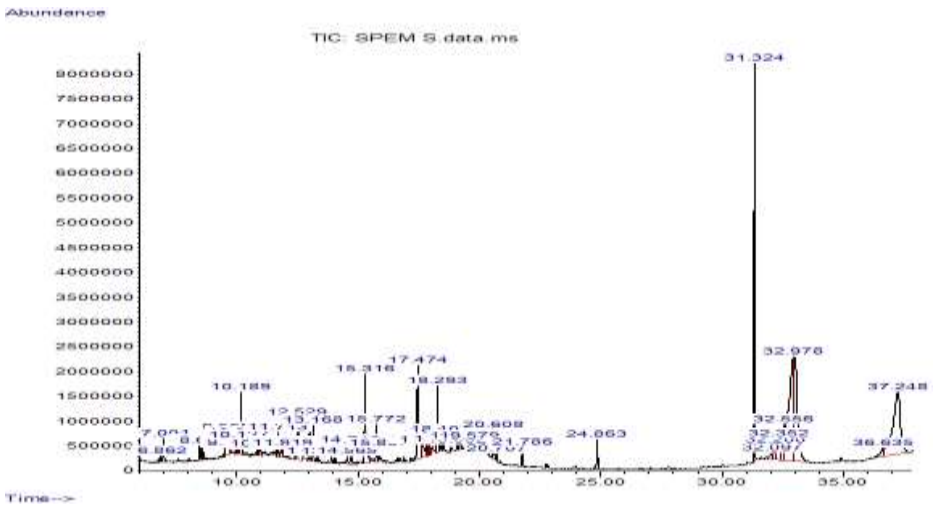

Figure 3.3. Result of GC-MS analysis of selected Saponin fraction of SPEM identified a total of 48 organic comounds with Succinic acid, 4-chloro-3-methylphenyl 2-methoxyphenyl ester (31.56%) and Propanamide, N-(3-methoxyphenyl)-2,2-dimethyl- (31.23%) predominant according to the peak areas.

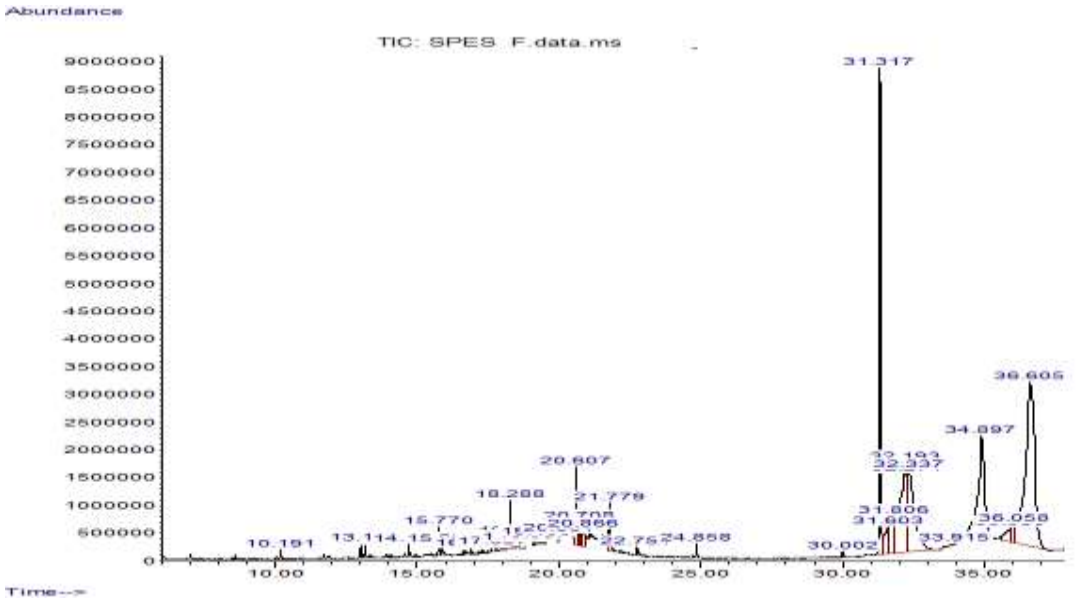

Figure 3.4. Result of GC-MS analysis of selected Flavonoid fraction of SPES revealed the presence of 32 organic compounds with Cyclotrisiloxane, hexamethyl- (46.98%) and 1,2-Benzisothiazol-3-amine, TBDMS derivative (36.57%) predominant according to the peak areas.

The LD_{50} was calculated as the geometric mean of the maximum dose that did not cause any death and the minimum dose that caused total death. This gave an LD_{50} 1264.91mg/kg b.w for extract of SPEM-S (Succinic Acid) and was found to be non-toxic at doses ≤ 1000 mg/kg b.w. as shown in Tables 2 and 3

Table 3.2. Result of LD_{50} for selected derivative of Saponin fraction of SPEM (Succinic Acid)

Phase 1				
Groups	**No. of Mice**	**Dose of Extract (Mg/kg)**	**No, of Deaths**	**No. of Survivals**
1	3	10	0	3
2	3	100	0	3
3	3	1000	0	3
Phase 2				
Groups	**No. of Mice**	**Dose of Extract (Mg/kg)**	**No, of Deaths**	**No. of Survivals**
1	3	1600	3	0
2	3	2900	3	0
3	3	5000	3	0

Table 3.3. Result of LD_{50} for selected derivative of Flavonoid fraction of SPES (Hexamethyl Cyclotrisiloxane)

Phase 1				
Groups	**No. of Mice**	**Dose of Extract (Mg/kg)**	**No, of Deaths**	**No. of Survivals**
1	3	10	0	3
2	3	100	0	3
3	3	1000	0	3
Phase 2				
Groups	**No. of Mice**	**Dose of Extract (Mg/kg)**	**No, of Deaths**	**No. of Survivals**
1	3	1600	0	3
2	3	2900	0	3
3	3	5000	0	3

4. DISCUSSION

In this study, the ethanolic extracts of SPEM, SPES were screened to identify the phytochemicals present in the extract and based on the colour intensities of each phytochemical components screened, six phytochemicals;

alkaloids, flavonoids, tannins, phenols, terpenoids, and saponins were selected for further study (See Table 3.1). The result was in line with (Agu & Okolie, 2017; Uniyal & Rahal, 2022; Usunobun *et al*, 2014) but at slight variance with (Nwaehujor et al., 2019) for *Annona muricata* (Soursop leaf).

Also, the quantitative analysis results of SPEM and SPES were in accordance with the phytochemical studies carried out (Chitra, 2021; Kazi et al., 2019) on *Mangifera indica* and (Nguyen, 2020; Nwaehujor et al., 2019) on *Annona muricata*, which showed high flavonoids, saponins, tannins and phenol contents (See Figure 3.1). But the result of the crude ethanolic yield was observed similar to that obtained (Aquisman *et al*., 2021) for mango leaves using 75% ethanol but slightly higher than that obtained by (Suhendar, 2019) for soursop in microwave assisted extraction (MAE) using 70% ethanol.

Moreso, yields for the phytochemicals selected for further study was similar for result on flavonoid and saponin for *Mangifera indica* but vary with results for phenol, tannin and alkaloid (Ebere Okwu & Ezenagu, 2008), and also varied for ethanolic extract of *Annona muricata* (Ugochi, 2018) except for saponin that was consistent (See Figure 3.2).

GC-MS analysis of the selected phytochemicals (Flavonoids and Saponins) for SPES and SPEM revealed the presence of organic compounds according to the mass spectral data and its closest hit in the NIST library (Valdez *et al*., 2018 (See Figure 3.3 and 3.4). Results are shown in Figures 3.3 and 3.4 for saponin fraction of SPEM and flavonoid fraction of SPES. The identified compounds are based the peak area (%), retention time (RT), molecular weight, molecular formular and interpretation based on National Institute of standard and Technique (NIST) library collection. Therefore, the peak area (%) of the most predominant derivative of the saponin fraction of SPEM was 39.74% whereas the flavonoid fraction of SPES gave the peak area (%) of 46.98% for its most predominant derivative. This led to the selection of Succinic Acid and Hexamethyl Cyclotrisiloxane as the potent derivatives of saponin fraction of SPEM and Flavonoid fraction of SPES respectively for isolation and future study.

Acute toxicity test (LD_{50}) was conducted on both samples in two phases and no death was recorded for SPES derivative (Hexamethyl Cyclotrisiloxane) in both phases and are thus concluded to be non-toxic at doses ≤ 5000mg/kg b.w. This was consistent with (Mbaeyi-Nwaoha *et al*., 2021). However, total death was recorded for SPEM derivative (Succinic Acid) in the second phase indicating that SPEM is only safe at doses ≤1000mg/kg b.w (See Table 3.2 and 3.3).

CONCLUSION

Although, plants contain various phytochemicals that are used as food supplements with inherent health benefits. The use of herbal mixtures for the treatment of diseases is wide spread; the side effects, dosage and route of administrations of these herbal remedies are poorly understood. We investigated the *in-vitro* acute toxicity (LD_{50}) of Hexamethyl Cyclotrisiloxane and Succinic Acid derivatives from ethanolic extracts of mango and soursop leave from the results, we conclude therefore, that both phytochemicals are safe for oral administration at low doses of about ≤ 1000 mg/kg b.w

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