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Phytochemical evaluation and antioxidant activity of *Zanonia indica l.* fruit extracts

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Abstract
Traditional practice for curing diseases using locally available medicinal plants is a culture adopted by Indians from prehistoric period. Cucurbitaceae members are also used as medicine for various sickness, one among them is *Zanonia indica* L. which is selected for the study. The aim of the investigation was to extract the phytochemicals and to examine the anti-oxidant property of the selected plant. Powder of dried fruit of *Z. indica* was subjected to Soxhlet extraction using different solvents to obtain crude extracts. Hexane, chloroform, acetone, methanol and distilled water was used for extraction. Crude extracts of these solvents showed the presence of phenols, alkaloids, terpenoids, flavonoids, steroids, carbohydrates, tannins, saponins, glycosides quinones, resins, and proteins in preliminary analysis. Quantitative analysis of crude extracts using HPLC showed that the acetone and methanol extract was rich in total alkaloids, total phenols, total flavonoids and total terpenes. Antioxidant activity of fruit extracts of Zanonia indica using DPPH assay, ABTS radical scavenging assay and FRAP assay revealed the presence of antioxidant property. Methanol and acetone extract of fruit was rich in antioxidants. This study evidenced the presence of bioactive compounds as well as its antioxidant properties and hence proves the traditional practice of the selected plant was appropriate.

Keywords: quantitative, cucurbitaceae, liana, phenols, percentage yield, antioxidant, methanol extract

Introduction
Tribal people in India practice folk medicine and utilize and the plants available in their area. The plants at their natural habitat underwent stress and resulted in production of many metabolites which are having immense therapeutic value and can be extracted and utilized as medicine. The fruit of *Zanonia indica* L. also used to cure various diseases like stomach discomforts [1]. Water is filled inside the empty fruit and taken for fever, stomach-ache and urinary problems [2]. Fruit is aperient, laxative and have cooling effect and cures asthma [3]. *Zanonia indica* L. is a cucurbitaceae member found growing near the river streams [4]. It is a climber with long flexible stem, usually twines around other tree and grows with the support and hence it behaves as a liana. Leaves of *Zanonia indica* L. is simple large and dark green, fruit is 8cm to 10cm long, pendulous and stated as bandolier fruit because of its arrangement [5]. Seeds are 5-6 in number, with papery wings they are 5.5cm to 6.5cm long, 1cm wide and grayish white in color [6]. Fruits usually seen during the month of December to February. Extraction of active ingredients and phytochemical analysis of fruit of *Zanonia indica* L. has been carried out in this study and anti-oxidant properties have been investigated.

Methodology
Collection and authentication of plant sample
The healthy fresh whole fruits were collected from Alevooru, Udupi district Karnataka, during the month of February 2020. The plant herbarium specimen was authenticated by Raja Kullayi Swamy, Indian Institute of Science, Bengaluru. Voucher specimen was conserved under the reference number HJCB1348 in JCB herbarium centre.

Extraction of plant sample
Fruit samples of *Z. indica* were collected and brought to the laboratory, washed with tap water and distilled water. Samples are cut into small pieces using a sterile scalpel and shade dried for about 30 to 40 days. The dried fruit samples were mechanically powdered and 100grams of powder was extracted in a Soxhlet apparatus using successive extraction method with 1000ml of different solvents of increasing polarity *viz*; hexane, chloroform, acetone, methanol, and distilled water for 24 hours each. Solvents were separated using distillation apparatus. All the extracts were evaporated to dryness at room temperature and stored in airtight glass bottles and kept in the refrigerator [7].

Extraction Yield
Extraction yield was analyzed by successive extraction method using solvents with increasing polarity (hexane, chloroform, acetone, methanol and water).
Percentage yield of the plant extract was calculated using the formula [7].

$$\% \text{ Yield of the extract} = \frac{\text{Weight of dried crude extract in grams}}{\text{Weight of plant sample powder in grams}} \times 100$$

Qualitative Phytochemical Analysis
The *Zanonia indica* L. fruit hexane extract (ZFHE), Zanonia fruit chloroform extract (ZFCE), Zanonia fruit acetone extract (ZFAE), Zanonia fruit methanol extract (ZFME) and Zanonia fruit distilled water extract (ZFWE) were subjected to various qualitative tests to determine phytochemical constituents [7, 8].

Quantitative Phytochemical Analysis
The total alkaloids, total phenols, total flavonoids, total terpenes were analyzed using high performance liquid

chromatography (HPLC) (Waters model no. 486; Waters Corp., Milford, MA, USA) on C18 column (symmetry, 4·6mm×250mm) in isocratic mode. The mobile phase methanol and water was used in the ratio 7:3 with the RP-HPLC C-18 column at a flow rate of 1mL/min. Before using, solvents were filtered with membrane filter and then sonicated for 15min [9].

HPLC analysis

The caffein, gallic acid, rutin, and tannic acid was used as standard for alkaloids, phenols, flavonoids and terpenoids respectively with the concentration 0.1mg/mL. Sample (1mg/mL) were dissolved in mobile phase and 20µL was injected and the elution was monitored at 230nm, 254nm, 272nm, 210nm respectively [7]. The amount of total alkaloids, total phenols, total flavonoids and total terpenoids present in the sample was estimated using the formula,

$$\frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{standard amount}}{\text{dilution of standard}} \times \frac{\text{dilution}}{\text{sample amount}} \times \text{Mean weight}$$

Anti-Oxidant Activity

The antioxidant properties of the fruit extracts were determined using DPPH, ABTS radical scavenging assay and FRAP assay method.

Determination of DPPH free radical-scavenging activity:

The potentiality of extracts to scavenge 1-1 -diphenyl - 2-picrylhydrazyl radicals were determined by preparing Various concentrations of fruit extracts of the sample with methanol and the addition of 3ml of 0.1 mM DPPH (3.94mg of DPPH in 100ml methanol). The solution was incubated in dark at room temperature for 15 min [10]. Absorbance was measured at 517 nm using a spectrophotometer [11]. Gallic acid is used as a standard. Reagent DPPH reagent was used as control. Percentage inhibition values of each concentration were calculated from the absorbance of the control (Ac) and absorbance of the sample (As) using the equation (1). IC_{50} values were determined using a graphical equation.

$$\% \text{ inhibition} = [Ac - As/Ac] \times 100 \quad (1)$$

Determination of ABTS radical scavenging activity

The potentiality of extracts to scavenge 2,2-Azino-bis, 3-ethylbenzthiazoline- 6-sulphonic acid (ABTS) radicals were determined by preparing Various concentrations of fruit extracts of the sample with methanol and addition of 3ml of ABTS. The solution was incubated in dark at room temperature for 30 min [10]. Absorbance was measured at 734nm using a spectrophotometer [12]. Before the assay ABTS reagent was prepared by 7mM of ABTS in distilled water with 2.4mM potassium persulfate kept in dark at room temperature for 15hrs. ABTS Reagent was used as control. Gallic acid is used as a standard. Percentage inhibition values of each concentration were calculated from the absorbance of control (Ac) and absorbance of sample (As) using the equation (1). IC_{50} values were determined using a graphical equation.

Determination of FRAP assay

FRAP (Ferric Reducing antioxidant power) assay was done by preparing different concentrations of fruit extracts of the sample with methanol and 2.5ml of 0.2M phosphate buffer with 6.6 PH along with the addition of 2.5ml of potassium ferricyanide. The mixture was Incubated at 50ºc for 20min and then 2.5ml of 10% TCA (Trichloroacetic acid) was added. The solution was Centrifuged at 3000rpm for 10min and 2.5ml of the upper layer was transferred to an empty test tube. 2.5ml of distilled water and 0.5ml of 0.1% ferric chloride were added and the absorbance was measured at 700nm in a UV visible spectrometer [15]. Gallic acid is used as a standard.

Results and Discussion

Collection and extraction of fruit sample

Shade dried Fruit samples were powdered and extracted in Soxhlet apparatus using different solvents such as hexane, chloroform, acetone, methanol and distilled water. The crude extracts containing bioactive compounds were get separated according to solvent polarity. Each solvent extract containing different bioactive compounds was evaluated through qualitative analysis.

Extraction Yield

The extraction yields of different solvent extracts are measured. Among the solvents, distilled water resulted in the highest extraction yield (8.9%), followed by methanol (3.4%), acetone (3.2%), chloroform (2.5%), and hexane (2.4%). The physical status and percentage yield of the extracts are as specified in table 1.

Table 1: Physical status and percentage yield of the Fruit extracts

Sl no.	Fruit extracts	Quantity of fruit sample and solvent used for extraction		Physical status	Yield in grams	% yield
		Powder(gm)	Solvent(ml)			
1	ZFHE	100	1000	Light green	2.4	2.4
2	ZFCE	100	1000	Dark green	2.5	2.5
3	ZFAE	100	1000	Yellow	3.2	3.2
4	ZFME	100	1000	Yellow	3.4	3.4
5	ZFWE	100	1000	Dark brown	8.9	8.9

Qualitative phytochemical analysis

Bioactive chemicals have a medicinal property and are extracted using different solvents. It can be evaluated by qualitative phytochemical analysis. *Zanonia indica* L. fruit extracts showed the presence of phytochemicals such as phenols, flavonoids, terpenoids, alkaloids, steroids, saponins, proteins, carbohydrates, tannins, quinones. The results represented in table 2 reveals the presence of Flavonoids, carbohydrates, proteins, and glycosides were present in all the extracts. Phenols were present in ZFAE, ZFME and ZFWE. Alkaloids were absent in ZFWE and ZFHE. In total ZFWE, ZFAE and ZFME contain more bioactive ingredients. These bioactive compounds contain many pharmacological properties such as antimicrobial, antidiabetic, anti-inflammatory, antioxidant, anti-cancerous and hence it is essential to analyze their specific activity [13].

Table 2: Qualitative phytochemical analysis of *Z. indica* fruit extracts

Sl.no	Qualitative Tests		Fruit extracts				
			ZFHE	ZFCE	ZFAE	ZFME	ZFWE
1	Test for Alkoloids	Hager's test Wagner's test Mayer's test	---	+++	+++	+++	---
2	Test for Flavonoids	Lead acetate test Ferric chloride test Alkaline test	++	++	+++	+++	+++
3	Test for Phenols	Lead acetate test Ferric chloride test	--	--	++	++	++
4	Test for Terpenoids	Copper acetate test Salkowski test Liebermann- Burchard test	---	++	+++	+++	+++
5	Test for Tannins	HCl test Braymer's test	--	--	++	++	++
6	Test for Carbohydrates	Fehling's test Benedict's test	++	++	++	++	++
7	Test for Saponin	Honey comb foam test	+	+	-	-	+
8	Test for Quinones	HCl test	-	+	+	+	+
9	Test for Resins	Turbid test	+	+	-	-	-
10	Test for Glycosides	Keller-Kiliani Test	+	+	+	+	+
11	Test for Proteins	Biuret test	+	+	+	+	+

Positive (+) and negative (-) signs indicate the presence and absence of phytochemical compounds respectively. More + sign indicates number of tests conducted with presence of bioactive compounds.

Quantitative phytochemical analysis

Total bioactive compounds present in the crude extract were analyzed by Quantitative phytochemical analysis using High-Performance liquid chromatography (HPLC). The amount of metabolite is proportional to the height and area of the peak in the chromatogram, and it was calculated manually. Quantitative analysis of total bioactive compounds such as alkaloids, phenols, flavonoids, and terpenes using HPLC is represented in chart 1 and the amount of these compounds extracted from different solvents in terms of mg/g are depicted in table 3. Acetone extract (ZFAE) and methanol extract (ZFME) were rich in all bioactive ingredients. Flavonoids and terpenes extracted more in ZFAE 130.78mg and 163.68mg respectively.

Alkaloids were nil in ZFWE. ZFWE is rich in phenols (49.66mg) than all other extracts. ZFHE contains a very less amount of metabolites among other extracts. All extracts were rich in flavonoids and terpenes. Terpenes show antioxidant, anti-inflammatory, antidepressant, antibiotic, anti-cancerous properties and flavonoids show antimicrobial, anti-inflammatory and antioxidant properties and act as free radical scavengers. Figure 1, 2, 3, and 4 are the HPLC chromatogram of total alkaloids, total phenols, total flavonoid, and total terpenes respectively.

Table 3: Quantitative phytochemical analysis of *Z. indica* fruit extracts by HPLC

Fruit Extracts	Amount in mg/g of sample			
	Alkaloids	Phenols	Flavonoids	Terpenes
ZFHE	4.94	3.670	11.977	0.603
ZFCE	19.15	6.68	27.88	64.507
ZFAE	33.400	18.558	130.789	163.684
ZFME	22.317	12.582	94.937	110.110
ZFWE	-	49.665	71.510	67.092

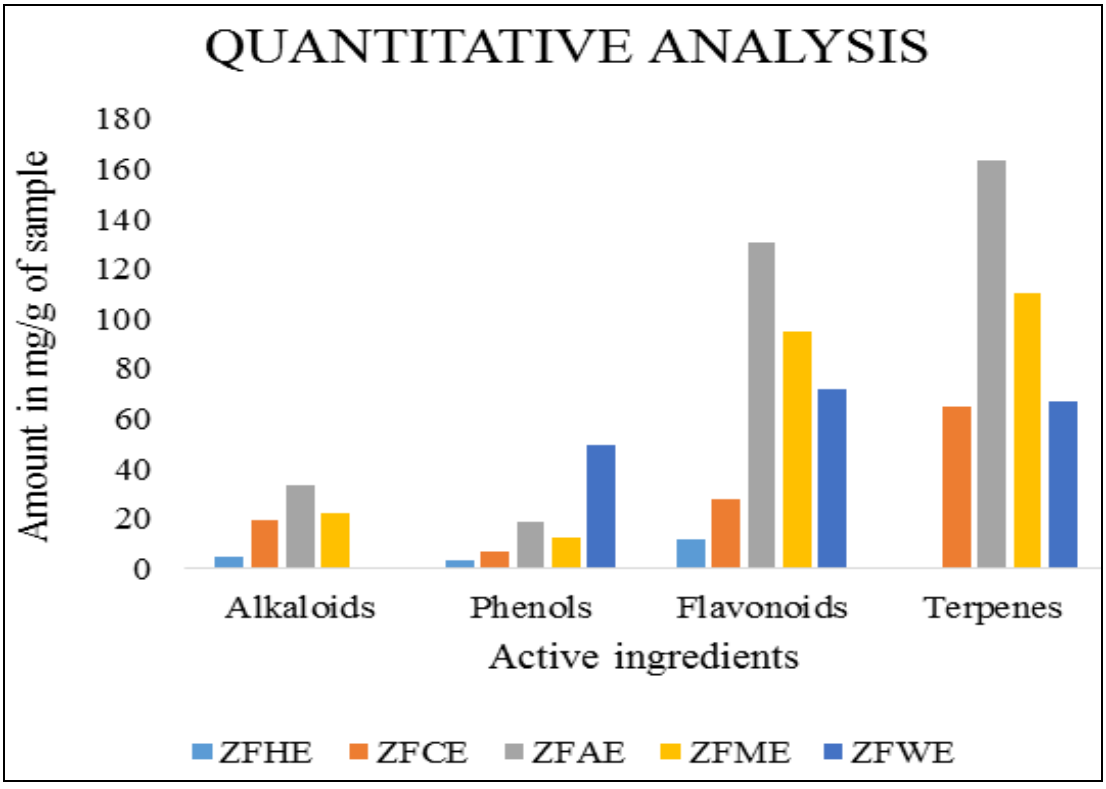
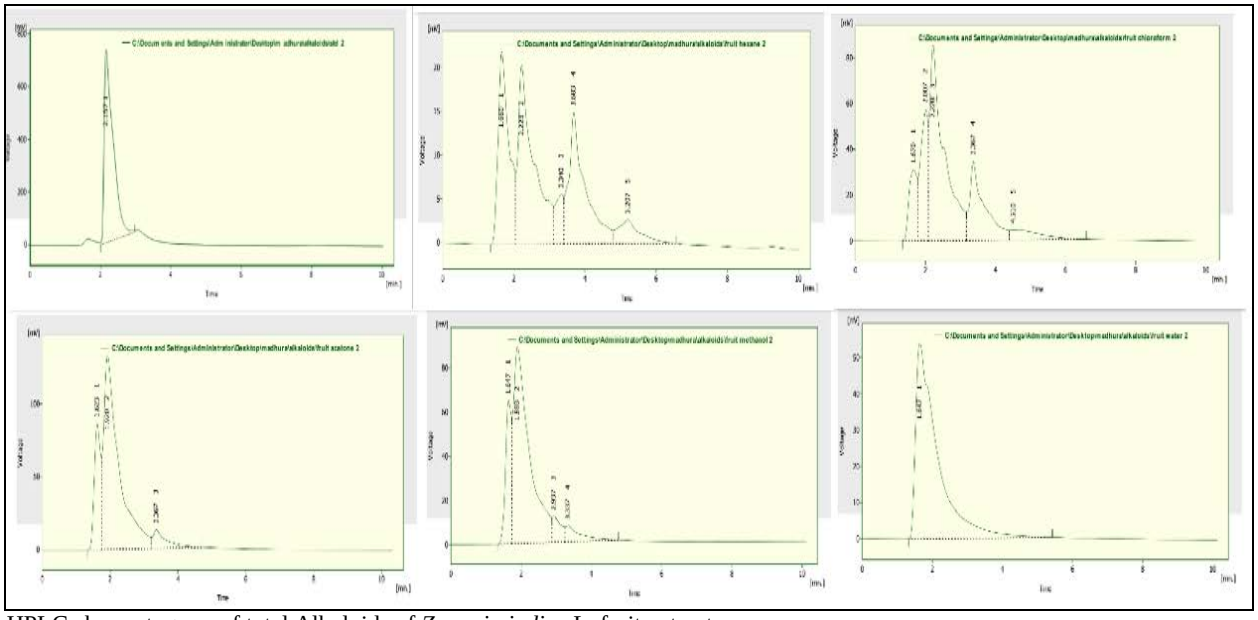
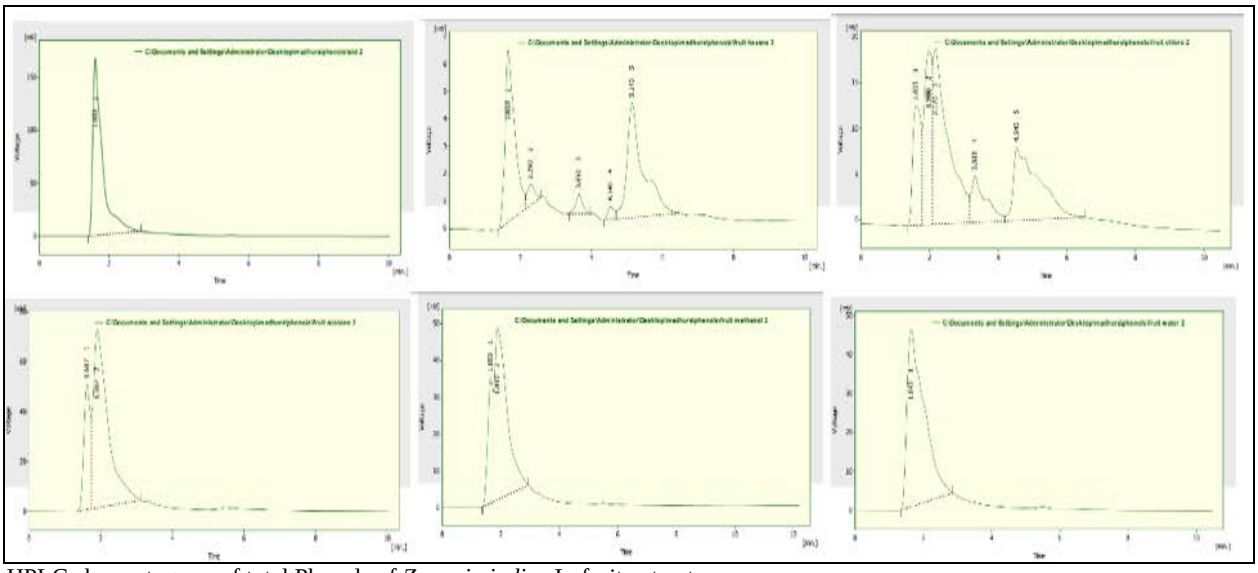


Chart 1: Amount of Total alkoloids, phenols, flavonoids and terpenoids present in different solvent extracts of *Zanonia indica* L. fruit



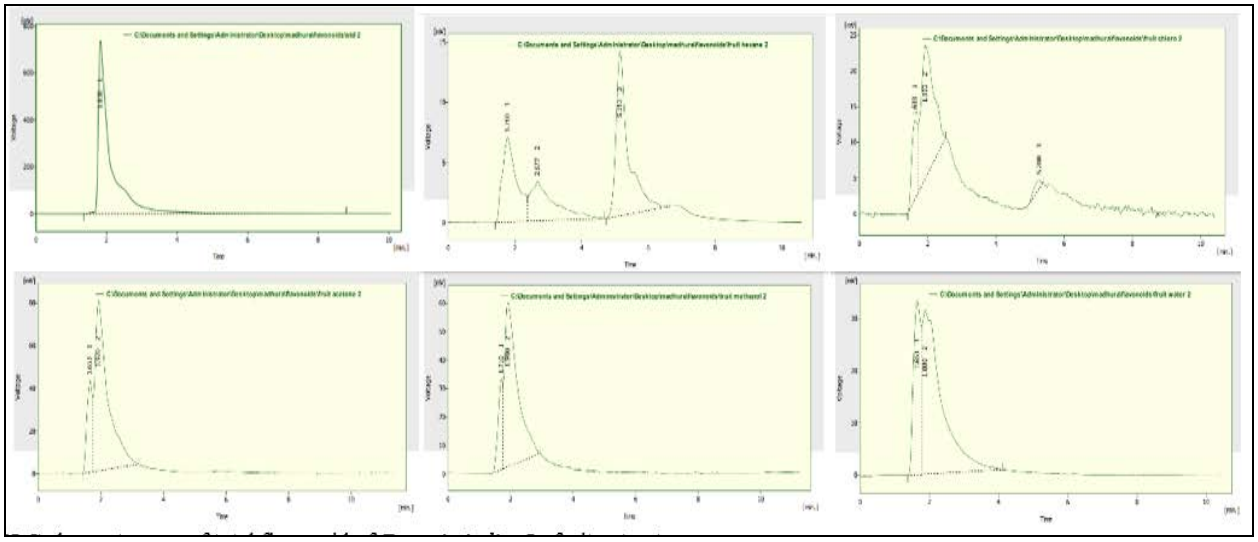
HPLC chromatogram of total Alkoloids of *Zanonia indica* L. fruit extracts

Fig 1: HPLC analysis of total alkaloids



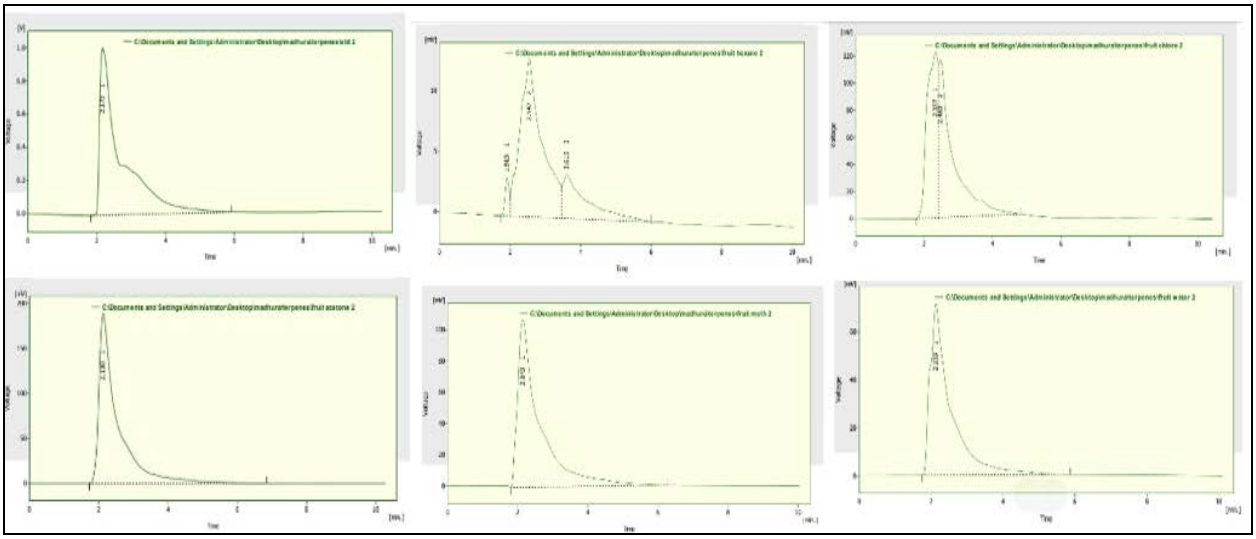
HPLC chromatogram of total Phenols of *Zanonia indica* L. fruit extracts

Fig 2: HPLC analysis of total phenols



HPLC chromatogram of total flavonoid of *Zanonia indica* L. fruit extracts

Fig 3: HPLC analysis of total flavonoids



HPLC chromatogram of total terpenes of *Zanonia indica* L. fruit extracts

Fig 4: HPLC analysis of total terpenes

Anti-Oxidant Activity

DPPH assay

DPPH is characterized as stable nitrogen cantered free radical, delocalized spare electrons give violet color with methanol. When it receives hydrogen atoms from the extract solution, it reduces its violet color to yellow. The extracts which are capable of performing this reaction is considered as an antioxidant [14]. The absorbance data were recorded against the selected concentrations. Graph plotted in excel, inhibition percentage on y-axis and concentrations on x-axis is represented in chart 2. Half maximal inhibitory concentration (IC50) values for standard (Gallic acid) and the fruit extracts were inscribed in the table (4). When extracts were compared to the standard with IC50 value (8.048 µg/ml), ZFAE and ZFME showed 176.1µg/ml and 329.01µg/ml respectively and showed good antioxidant activity compared to other extracts.

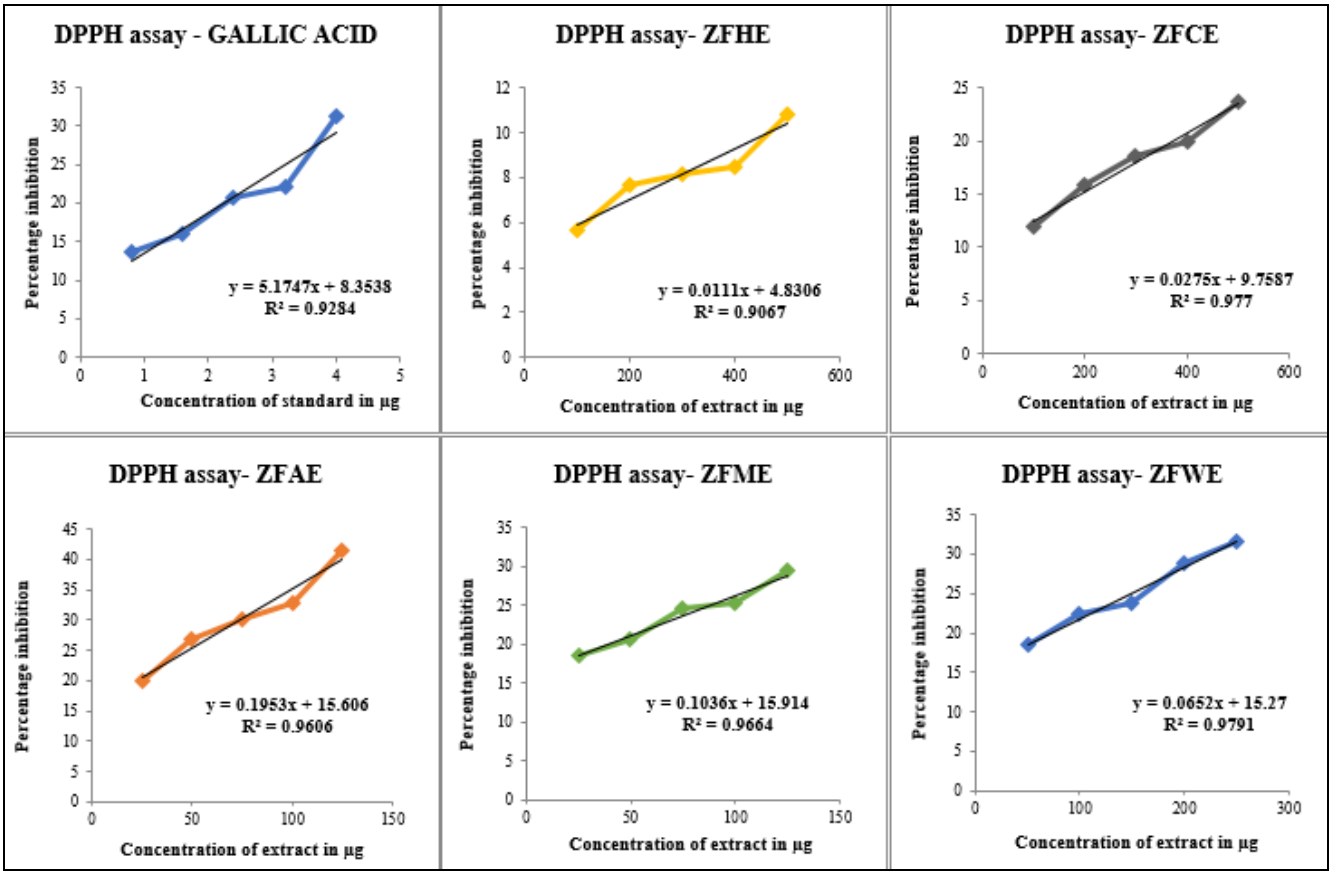


Chart 2: DPPH Scavenging activity of fruit extracts of *Zanonia indica*

ABTS assay

ABTS forms a stable radical upon oxidation of one electron. This mixture is used to determine the total antioxidant capacity. ABTS radical is reactive towards most antioxidants present in the crude extract and change blue

color to green. The reaction is monitored spectrophotometrically at 734nm. The absorbance data were recorded against the selected concentrations. Graph plotted in excel, inhibition percentage on y-axis and concentrations on x-axis is represented in chart (3). Half maximal inhibitory concentration (IC50) values for standard (Gallic acid) and the fruit extracts were inscribed in the table (4). When extracts were compared to the standard (1.071µg/ml) with IC50 values, ZFAE, ZFME and ZFWE showed 43.63µg/ml, 44.36µg/ml and 138.48µg/ml respectively and showed good antioxidant activity compared to other extracts.

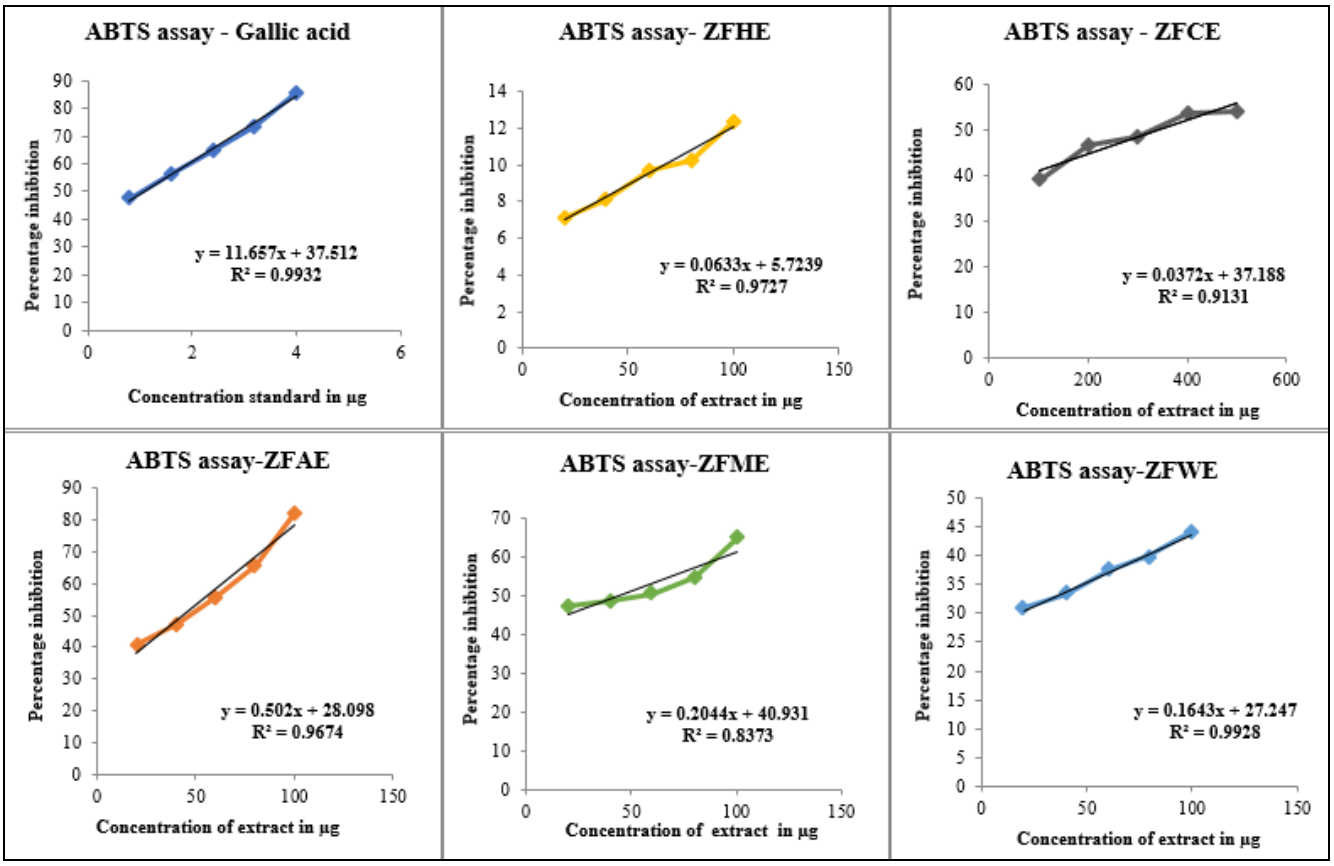


Chart 3: ABTS Scavenging activity of fruit extracts of *Zanonia indica*

Table 4: IC50 values of Gallic acid and the fruit extracts

Fruit Extracts	Ic50 of DPPH	Ic50 of ABTS
Gallic acid	8.048	1.071
ZFHE	4069.3	699.46
ZFCE	1463.32	344.4
ZFAE	176.1	43.62
ZFME	329.01	44.36
ZFWE	532.66	138.48

FRAP assay

Ferric Reducing antioxidant power assay has been carried out on the fruit extracts of *Zanonia indica.* Depending on the presence of active ingredients in the extract, it has been shown that it has a reduction potential by acquiring electrons and reduced potassium ferricyanide (Fe3+) to potassium ferrocyanide (Fe2+). Further reacting with ferric chloride, it formed a ferric-ferrous complex and turn the solution colorless to intense blue color. Higher absorbance indicates a higher ferric reducing power. The fruit extracts of Zanonia showed increased absorbance as the concentration increased. The absorbance at 700nm of specific concentration has been depicted in table (5). The values were plotted for absorbance against the concentration of extracts using excel is represented in chart (4). The chart clearly visualizes the ZFAE and ZFME have antioxidant properties when compared to standard gallic acid (GA). Gallic acid shows the absorbance of 1.189, ZFAE shows 1.119 and ZFME shows absorbance of 1.112 at 500 µg concentration.

Table 5: Absorbance values of FRAP at 700nm

CONC	GA	ZFHE	ZFCE	ZFAE	ZFME	ZFWE
100	1.041	0.748	0.789	0.812	0.85	0.801
200	1.09	0.769	0.88	0.919	0.936	0.902
300	1.1	0.781	0.92	0.985	0.951	0.939
400	1.13	0.799	0.95	0.993	0.971	0.962
500	1.189	0.82	0.989	1.119	1.112	0.995

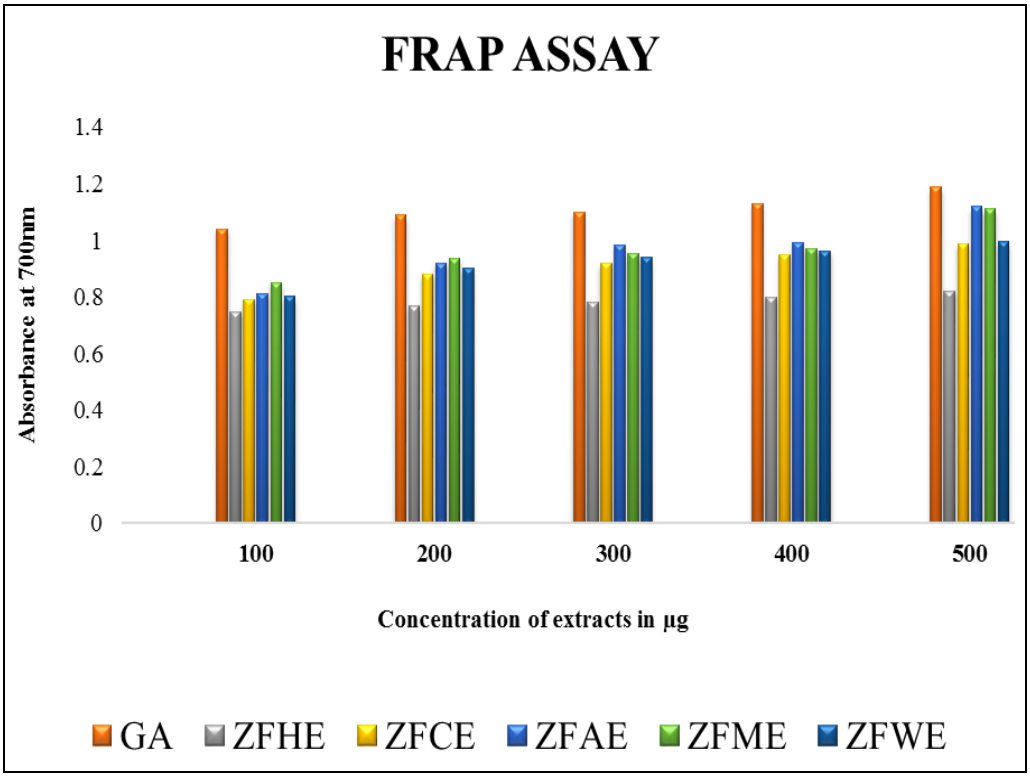


Chart 4: FRAP assay of fruit extracts of *Zanonia indica* L.

Conclusion

Phytochemical analysis of fruit extracts of *Zanonia indica* L. assured the presence of alkaloids, flavonoids, terpenes and phenols. These were rich in acetone extract and methanol extract. Extracts showed the best antioxidant property in all three assays. Among all extracts fruit acetone extract and fruit methanol extract was very active and showed positive results for antioxidant assay.

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Author's Contributions

The first author designed the study, experimented, and wrote the first draft of the manuscript. The Second author helped and supervised the project. Both authors read and approved the final manuscript.

Competing Interests

Authors have declared that no competing interests exist.

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