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Research Article

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Phytochemical Analysis, Antioxidant Assay and Cytotoxicity of Stem Bark Extracts of *Celtis integrifolia* L. (Cannabaceae)

JS Ezekiel*

Department of Chemistry, Faculty of Sciences, Abubakar Tafawa Balewa University, Bauchi, Nigeria

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ABSTRACT

The search for the phytochemicals that elicit antimicrobial and other pharmacological properties such as antioxidants and cytotoxicity is on the increase. Many species of the genus celtis have been found to possess medicinal values. C. africana, C. australis and C. occidentalis have been reported to contain phytochemicals that show some important pharmacological activities such as antioxidant and cytotoxic properties. Celtis integrifolia is used traditionally in the management of epilepsy, mental disorder and cancer. Phytochemical screening of the solvent extracts of C. integrifolia using standard methods revealed the presence of flavonoids, saponins, cardiac glycosides, terpenoids and tannins in varying proportions. The ethyl acetate(EA), acetone(AC), ethanol(EtOH) and methanol (MeOH) extracts gave good antioxidant properties of 52%, 65%, 64% and 57% inhibition of 2,2-diphenyl-1-picrylhydrazyl (DPPH) respectively. This is an indication that all of the solvent extracts except dichloromethane (DCM) contained antioxidant components. Similarly, when the EA and AC extracts were subjected to Brine shrimp lethality assay (BSLA), both fractions exhibited almost equal cytotoxicities. Both of the EA and AC extracts showed dose dependent ability to be cytotoxic. It killed about 50% of the Brine shrimp larvae, nauplii even at lowest concentration of 1 µg/ml. Therefore, the plant, C. integrifolia has proved to contain bioactive components that were antioxidant to DPPH as well as cytotoxic to Brine shrimp larvae, nauplii. This bioactivity shown by these solvent extracts from C. integrifolia is similar to research findings that led to the isolation of compounds having anticancer properties. Therefore, the traditional use of C. integrifolia to manage tumour and cancer could be due to the presence of the bioactive compounds detected in the stem bark of the plant.

Keywords: Phytochemicals; Antioxidants; Cytotoxicity; Anticancer; Bioactivity

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INTRODUCTION

The search for phytochemicals that elicit antimicrobial and other pharmacological properties such as antioxidant and cytotoxicity is on the increase. Plants that have been used traditionally to manage a particular ailment have been shown to possess phytochemicals responsible for such effects [1]. Found Cadaba farinosa Forsk which is traditionally used for the management of breast cancer to elicit antioxidant and cytotoxic properties. Plants showing antioxidant and cytotoxic properties have previously yielded anticancer compounds [2]. Also plant species belonging to the same genus or family with the plants that are known to contain useful phytochemicals have been shown to possess similar useful constituents [3,4]. It is against these backdrops that the current research was conducted.

The plant genus *Celtis* commonly known as hackberries is a genus of about 60-70 species of deciduous trees widespread in warm temperate regions of the Northern Hemisphere, [5]. Many of the species of the genus celtis have been found to possess medicinal values. *C. Africana*, *C. australis* and *C. occidentalis* have been reported to contain phytochemicals that show some important pharmacological activities such as antioxidant and cytotoxic properties [4,3,6]. *C. integrifolia* is commonly found in Nigerian savannah and is implicated in the management of epilepsy [7], mental disorder, weakness and sore throat [8], cancer, wound healing, spices and aphrodisiac in northern Nigeria [7] have reported the presence of proline, sugar, gallic acid, leucocyanidin and gama amino butyric acid (GABA) in the stem bark extract of *C. integrifolia*. There is a need to research more on the plant to unravel the underlying medicinal potentials as reported in the ethnopharmacology of the plant.

MATERIALS AND METHODS

Identifiable parts of *Celtis integrifolia* L. (Cannabaceae) were collected from a Savannah region, of Nigeria, W/Africa and conveyed to North East Arid Zone Development Programme (NEAZDP), Gashua,Yobe State, Nigeria where identification was duly made by a plant taxonomist.

The stem bark of the plant was collected and shade dried. The dried material was pulverized in a clean mortar and pestle into coarse powder. The pulverized plant materials were subjected to exhaustive sequential soxhlet extraction. Solvents with varying polarities were used for the extraction which started with solvent of lower polarity, n-hexane, followed by dichloromethane, ethyl acetate, acetone, ethanol and aqueous methanol (1/1 v/v). 100 g of sample was extracted for 6 hours with each of the various solvents. Six different extract fractions (HX, DCM, EA, AC, EtOH and MeOHaq) were separate from the different solvents used and concentrated on rotary evaporator and evaporated at atmospheric conditions.

PHYTOCHEMICAL SCREENING

Phytochemical analysis was performed on the crude extracts using the standard methods of [9-11]. The phytochemicals that were screened for are: alkaloids, flavonoids, saponins, tannins, cardiac glycosides, anthraquinones, steroids, terpenoids, reducing sugar and phenolics.

ANTIOXIDANT ASSAY

The capacity of the extracts to scavenge the stable free radical, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), was monitored according to [14,15]. From each extract solutions (1 mg/ml in 95% methanol), 2 ml were taken and mixed with 2ml of 0.1 mM methanolic solution of DPPH free radical. This reaction was conducted in triplicate for accuracy of experiment. The mixtures were vortexed thoroughly for one minute at room temperature and incubated for 30 minutes in the dark. Finally, absorbances of each of the mixtures were read at 517nm on a JENWAY 6305 UV-spectrophotometer. The same experiment was conducted with a negative control or blank constituted of 95% methanol mixed with the DPPH solution and with a positive control constituted of 1mg/ml of L-(+)-Ascorbic acid dissolved in 95% methanol. The antioxidant capacity of each extract sample was expressed in terms of percentage inhibition and was calculated as:

$$\%\text{Inhibition} = \frac{Ab - Ac}{Ac} \times 100,$$

Where, Ab=the absorbance of blank,

And Ac=absorbance of extract or control.

CYTOTOXITY

Brine Shrimp Lethality Assay (BSLA)

In a general primary screening for bioactivity, the popular bioassays used include the brine shrimp lethality assay (BSLA) and the crown-gall tumour inhibition test [12-16]. The first technique is a bench-top screening method and, was adopted in this research. It is an *in vivo* lethalilty test using a tiny crustacean, which is the brine shrimp (*Artemia salina*). Since the introduction of this technique in 1982, this test has been used for the isolation of *in vivo* antitumour agents and pesticides from plant [16].

In this technique, eggs of the brine shrimp were added to a hatching chamber containing artificial sea water, the chamber was kept under inflorescent lamp for 48- 72hrs for the eggs to hatch into shrimp larvae, that is nauplii. From the *Celtis integrifolia* stem bark extracts, 20 mg were taken and separately dissolved in 2 ml of methanol, to serve as stock solution1. From these stock solutions, several dilutions were prepared. Each test sample has 4 serial dilutions to gives the concentrations of 20 mg/2ml, 2 mg/2 ml, 0.2 mg/2 ml and 0.02 mg/2 ml which correspond to 1000 ppm, 100 ppm, 10 ppm and 1 ppm, respectively. A control (blank) solution was made by taking 5ml of the artificial sea water in a sample vial and a drop of DMSO. All the sample vials containing 0.5ml of the various sample concentrations were allowed to completely evaporate. Each sample was prepared in triplicate and to each of the evaporated sample vial; 4.5ml of artificial sea water was introduced by use of pipette followed by a drop of DMSO to hasten solubilization of test samples in the vials. Ten free-swimming nauplii were introduced into each vial by use of Pasteur pipette and the total volume of the sea water was adjusted to 5ml by dropping in more of the sea water from a dropper and allowed to stand for 24 hours after which the number of free-swimming nauplii were

counted and the LC50 or percentage (%) mortality was computated to give the final cytotoxicities of the test extracts [17-19].

LC50 is the lethal concentration at which 50% of test organisms were killed and this can also be determined by calculating the percentage mortality from which the lethal concentration can also be determined. LC50 of less than 100µg/ml was considered as potent (active), but according to Lilybeth et al. [18], LC50 less than 1000µg/ml is toxic while LC50 values greater than 1000µg/ml is non-toxic. The percentage mortality (%M) was also calculated by dividing the dead nauplii by the total number of nauplii used and then multiplied by 100. This is to ensure that the death (mortality) of the nauplii is attributed to the bioactive compounds present in the plant extracts.

RESULT

Phytochemical screening of the solvent extracts of *C. integrifolia*.

The results of phytochemical screening were presented in the Table below showing presence of different types of bioactive secondary metabolites in various solvents.

Table 1: Result of phytochemical screening of the solvent extracts from *C. Integrifolia*

Phytochemicals/Reagents	Solvent extracts				
	DCM	EA	AC	EtOH	MeOH (aq)
Alkaloids	-	-	-	-	-
Flavonoids	-	+	++	++	+
Saponins	++	-	-	-	-
Cardiac glycosides	++	+	+	+	+
Steroids	-	-	-	-	-
Terpenoids	-	+	++	-	-

DPPH Free Radical Scavenging Capacity of *C. integrifolia* Crude Extracts

All of the extracts from *C. integrifolia* were subjected to antioxidant assay using DPPH stable free radical. All of the extracts except DCM showed moderate antioxidant properties expressed in percentage inhibition. The result has been presented in the chart shown below.

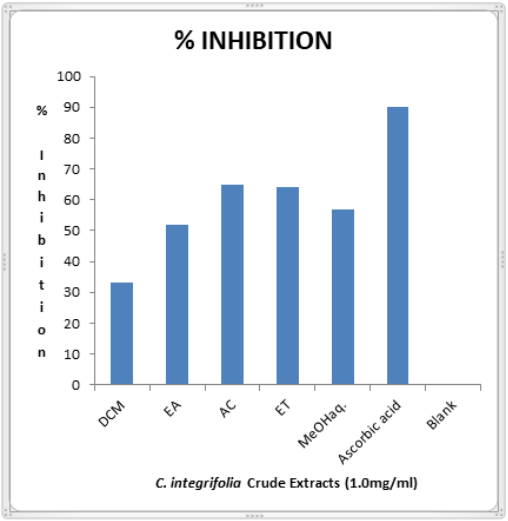

Figure 1: Chart showing the Percentage inhibition of DPPH by the *C. integrifolia* extracts

Brine Shrimp Lethality Assay (BSLA)

The Brine shrimp lethality assay (BSLA) of ethyl acetate and acetone extracts of *C. integrifolia* were recorded and plotted on a chart shown below. The chart presents the percentage mortality of brine shrimp larvae when treated with AC and EA of *C. integrifolia* extracts.

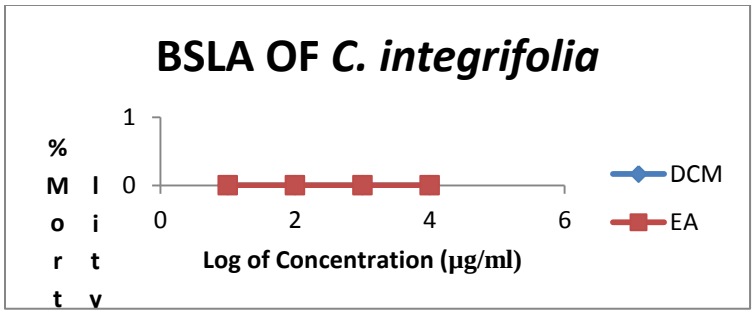

Figure 2: Chart showing result of Brine shrimp lethality assay of EA and AC

DISCUSSION

Phytochemical Screening

Table 1 presents the results of the phytochemical screening of the solvent extracts of *Celtis integrifolia L*. (Cannabaceae). Phytochemical screening of dichloromethane (DCM), ethyl acetate (EA), acetone (AC), ethanol(EtOH) and aqueous methanol (MeOHaq) extracts of *C. integrifolia* showed the presence of alkaloids, flavonoids, cardiac glycosides, saponins and terpenoids. Alkaloids are common chemicals in medicinal plant, but they are often toxic at high concentration, and many have dramatic physiological and neurological activities. Tryptamine alkaloids cause staggering gait and death while, tropane alkaloids such as hyoscyamine consumption causes paralysis and rapid heartbeat leading to death [10,20]. Many alkaloids such as strychnine and coniine are poisonous whereas others are used in medicine in controlled concentration as pain relievers, antitussive like morphine and codeine more especially the steroidal alkaloids [21,22]. The alkaline and lead acetate test revealed the presence of flavonoids in almost all of the extracts of both plants except in DCM extracts. Researchers have repeatedly ascribed antioxidant properties of medicinal plant extracts to flavonoids and phenolic compounds. Perveen et al., El-Alfy et al. and Alzeer et al. [2,4,23] isolated flavonoids that were responsible for the antioxidant and cytotoxic properties of the plants' extracts. El-Alfy et al. and Mata et al. [4,24] reported the isolation of anticancer flavonoids through bioassay guided isolation of antioxidant and cytotoxic compounds. Flavonoids have been shown to have a wide range of biological activities including; antiallergic, antibacterial, anti-inflammatory, antimutagenic, antioxidant, antiproliferative, antithrombotic, antiviral, hepatoprotective and antihypertensive [2,22,25].

Frothing and emulsion tests detected saponins in all of the extracts. Terpenoids were slightly detected in EA, and more in AC extracts but not in DCM, EtOH and MeOH (aq) extract. Plant terpenoids exhibit various pharmacological activities such as anti-inflammatory, anticancer, antimalarial, inhibition of cholesterol synthesis, antiviral and antibacterial activities [26]. Terpenoidal saponins isolated from Platycodon grandiflorum inhibited hepatitis C virus replication [27]. The extracts did not show presence of steroids, anthraquinones, reducing sugar and phlobatannins. Anabolic steroids have been observed to promote nitrogen retention in osteoporosis and animals with wasting illness [22]. The European Foods and Safety Authority(EFSA) concluded that blood cholesterol can be reduced on average by 7-10.5% if a person consumes 1.5-2.4 grams of plant sterols and stanols every day. Therefore, blood cholesterol lowering may reduce the risk of coronary heart disease [28]. Tannins were shown to be present in little quantities in all but DCM of the extracts of *C. integrifolia*. The antimicrobial activities of tannins are well documented. Tannins are known to inhibit pathogenic fungi [22]. The growth of many fungi, yeast, bacteria and viruses were inhibited by tannins. Tannins have also been reported to exert other physiological effects such as to accelerate blood clotting, reduce blood pressure, decrease serum lipid level, produce liver necrosis and modulate immunoresponse [29]. Plant tannins have also been shown to provide a novel therapeutic option for major factors in the induction of ulcerative colitis [30,31]. Hydrolysable tannins are potentially toxic to animals. Consumption of feeds containing high levels of hydrolysable tannins causes liver and kidney toxicity and lead to death of animals [20]. Anthraquinones are considered to be one of the most active agents in treatment of metastatic breast cancer [22]. Most plants containing anthraqinones possess laxative properties. The consumption of glycosides and their derivatives in anthraquinone containing plants determine their effectiveness as laxatives [25]. The derivatives of anthraquinones present in purgative drugs may be dihydroxy phenols and trihydroxy phenols such as chrysophanol and emodins respectively [10]. Anthraquinones were not detected in both plants.

ANTIOXIDANT ASSAY

The scavenging activities of the crude extracts of *C. integrifolia* on 2,2-Diphenyl-1-picrylhydrazyl(DPPH) free radical are shown in Figure 1. The table displays the result for free radical scavenging property of *C. integrifolia* crude extracts. All extracts except DCM have percentage inhibition above 50% and this entails that the extracts possess antioxidant properties. In fact, methanol, ethanol and acetone have been found to be more effective in extracting antioxidant compounds from plants [12]. Asserts that the three solvents have been found to be good in extracting phenolic compounds and flavonoids with aqueous acetone being the best. However, it had earlier been reported by [12] that methanol particularly has been generally found to be more efficient in extracting lower molecular weight polyphenols while the higher molecular weight flavanols are better extracted with aqueous acetone, whereas ethanol is also a good solvent for the same purpose and safer for human consumption. A number of studies have reported the antioxidant and cytotoxic activity of some medicinal plants. Many compounds with antitumor activities were isolated based on only the antioxidant properties [2]. Reported the bioassay guided fractionation of active extract of *Chorizanthe diffusa* using DPPH free radical scavenging assay which led to the isolation of one novel compound (flavone) with antitumor property from ethyl acetate fractions. Also ethyl acetate soluble extract of the entire plant *C. diffusa* exhibited significant antioxidant activity based on the scavenging activity of DPPH. Table 1 showed the inhibitory properties of *C. integrifolia* extracts on DPPH which means that the extracts could contain bioactive components that elicit antioxidant properties and, perhaps that could be the reason why *C. integrifolia* is used traditionally to manage tumor, anemia and hemorrhage [8,7].

CYTOTOXITY

Brine shrimp lethality assay (BSLA) is an *in vivo* biological assay that has been used to select plants for antitumor and pesticide properties [16]. It has also been used to test the lethality of laboratory chemicals and consumer products [31]. From the present research, it is evident that two crude extracts of *C. integrifolia*(AC/CI and EA/CI) have shown to be cytotoxic to brine shrimp larvae(nauplii). The susceptibility of nauplii to the extracts was dose dependent (Figure 2). The cytotoxic effect of crude acetone and ethyl acetate extracts of *C. integrifolia* are not quite different. At high concentration(1000 µg/ml) percentage mortalities of both extracts were 87%. When diluted to 10 and 100 µg/ml, cytotoxicity of EA/CI became slightly higher but at lowest concentration of 1µg/ml AC/CI became slightly more cytotoxic of about 53% as against 50% of EA/CI. According to Khaled [19], LC50 below 1000 µg/ml(1000ppm) is cytotoxic and above that is considered inactive. Plants having LC50 values below 200 µg/ml(200ppm) are considered highly active. It is evident that mortality rate of 50% (Equivalent to LC50) ranges between 100 to 1µg/ml. This means that both extracts are highly active or cytotoxic to nauplii.

Brine shrimp Test(BST) has been reliably used to detect annonaceous acetogenins. Annonaceous acetogenins are powerful antitumor and pesticide products that are found only in the family Annonaceae. The novel acetogenin as well as annoglaucin were significantly active in brine shrimp test (BST) and were also cytotoxic for six human solid tumor cell lines [24]. Perhaps this explains why Muazu et al. [7] reported that *C. integrifolia* is used to manage tumors, by traditional healers.

CONCLUSION

Solvent extracts of *C. integrifolia* yielded flavonoids, saponins, cardiac glycosides, terpenoids and tannins in varying quantities. The bioactivity assay of the solvent extracts showed that the ethyl acetate, acetone, ethanol and methanol extracts exhibited moderate level of antioxidant properties on 2,2-Diphenyl-1-picrylhydrazyl(DPPH) stable free radical. Similarly, when the cytotoxicity of ethyl acetate and acetone extracts were experimented on Brine shrimp larvae, nauplii, the result showed that the two extracts were cytotoxic to the crustaceans. These bioactivities shown by the extracts of *C. integrifolia* is in line with several research on plant extracts that gave rise to isolation of anticancer agents. It can easily be said that the use of the plant traditionally to manage cancer by traditional healers in Nigeria is also in agreement with the current findings.

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