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Full Length Research Paper

Effects of lemon balm (*Melissa officinalis*) on behavioral deficits and memory impairment of rats surviving sepsis

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Sepsis has become one of the most frequent causes of mortality in intensive care centres. So far, there is no effective pharmacotherapy that can prevent or improve the neurological consequences and enhance survival. The goal of this study is to investigate the neuroprotective effect of lemon balm (*Melissa officinalis*) on behavioural dysfunctions produced in sepsis-surviving rats. Adult male rats were subjected to caecal ligation and puncture and the control animals were submitted to the sham operation. Lemon balm ethanolic extract or saline, given orally, was administered for one week after surgery procedures. Locomotion, anxiety, depressive behaviour and memory were investigated. In the elevated plus-maze (EPM), the percentage of open arm entries and open arm time was very significant in the animals treated with lemon balm extract, similar to the diazepam response in sham-operated and sepsis-surviving rats. Locomotion in open field tests and the enclosed arm entries in the EPM were not significantly altered by treatments. In the forced swimming (FS) test, the extract was effective at reducing the immobility time as that demonstrated by fluoxetine. In the step-down inhibitory avoidance test, the extract eased the effects on memory of sepsis-surviving animals. Collectively, these results demonstrate that lemon balm ethanolic extract could be used for the prevention of cognitive and mood-related deficits that may be associated with sepsis sequelae.

Key words: Anxiety, depression, locomotion, *Melissa officinalis*, memory.

INTRODUCTION

Brain disorders are currently getting top priority among the science target and the literature has shown the behavioural problems associated with traumatic brain injury. Sepsis is an example of this damage and it is characterized by a systemic inflammatory response and is associated with high mortality in humans (Greenberg et al., 2014). The main reason of death in septic patients could be encephalopathy (Mazeraud et al., 2016; Tauber et al., 2017). Nowadays, several studies are conducted to evaluate the damage to the brain during sepsis and its behavioural consequences, especially involving cognitive and mood impairments present in sepsis survivors (Comim et al., 2016; Leite et al., 2013; Mazeraud et al., 2016; Tauber et al., 2017). The polymicrobial sepsis induced in rats by caecal ligation and perforation (CLP) experimental models are important because they reproduce human sepsis consequences (Leite et al., 2013), which is clinically relevant for elucidating new therapy alternatives for this health problem (Hutchins et al., 2014).

Traditional medicine was used on people before the great development of modern medicine, based on multiple alternatives of natural products to treat several physiological dysfunctions. Research into the historical literature has demonstrated that some of the natural products and/or their pharmacologically active principles have a broad spectrum of biological activities, including central nervous system (CNS) properties (Andrade et al., 2016; Bu et al., 2016).

Piper nigrum L. (Piperaceae) used in traditional medicine of many countries, for example, has different properties, such as antiinflammatory, antioxidant, antidepressant and cognitive effects. The methanolic extract of their fruits in beta-amyloid rat model of Alzheimer's disease significantly exhibited anxiolytic- and antidepressant-like effects and also antioxidant potential (Hritcu et al., 2015). Acute treatment of rats with *Lindera obtusiloba* extracts, commonly used as an alternative medicine in Korea, significantly reduced immobility time and increased swimming time, suggesting that the antidepressant-like effects of this extracts were likely mediated via the glucocorticoid receptor (Lim et al., 2016). In addition, other neuroprotective efficacy could be observed in *in vivo* and *in vitro* experiments (Jalsrai et al., 2016).

Melissa officinalis is a medicinal plant from the Lamiaceae family that has been used as a folk medicine to threat central nervous disorders (Anheyer et al., 2017; Kennedy et al., 2002). This plant has neuroprotective activity at CNS because it displays potent antioxidant activity (Soodi et al., 2017). From the phytochemical point of view, the main chemical principles of this species of herbal medicine are flavonoids, alkaloids, phenolic acids and tannins (Noguchi-Shinohara et al., 2015; Shakeri et al., 2016). These compounds may contribute to the homeostasis of the CNS, especially because they can protect against neurodegeneration and/or behavioural disorders that can be correlated to dementia or traumatic brain injury. Notably, it has also been reported as lowering excitability, cognitive dysfunctions, stress and anxiety levels in rodent models and humans (Anheyer et al., 2017; Lin et al., 2015; Ross, 2015; Shakeri et al., 2016). Previous results from our lab showed that the oral intake of lemon balm EE for about 10 days has anxiolytic and antidepressant-like properties in a manner gender (male vs. female) dependent, particularly with regard to the effective dose for each exposure length (Taiwo et al., 2012).

Despite great availability of information regarding the neuroprotective effects of plant extracts, the options for sepsis treatment is scarce. In this context, the goal of this work is to verify the possible neuroprotective action of lemon balm extract on behavioural and cognitive deficits due to sepsis sequela in rats.

MATERIALS AND METHODS

Animals

Male Wistar rats (n= 60), 3 months old, 350 g, from the animal facility of Sena Aires Faculty (Valparaíso de Goiás-GO, Brazil), were housed 5 to a cage with food and water available *ad libitum* and were maintained on a 12-h light/dark cycle (lights on at 7:00 am) at room temperature (23 ± 1°C). The animals were allowed to adapt to the laboratory conditions for at least 1 week before the behavioural assessment. All procedures used in the present study complied with the guidelines on animal care of the UNB Ethics Committee on the Use of Animals, which follows the “Principles of laboratory animal care”. Subjects were transferred in their cages to the experimental room on the day of being tested, where they were left unbothered to habituate for at least 1 h before the behavioural tests.

Preparation of the plant extract

Dried leaves of *M. officinalis* were kindly supplied by Centroflora Group (Botucatu, Brazil), who also provided a certification of the plant’s identity and quality. Powdered plant material (1900 g) was extracted by maceration at room temperature (24±3°C) using ethanol as solvent. Following removal of the solvent, under vacuum conditions and below 40°C using a Heildolph system (Heildolph ® Instruments, Germany) composed by a rotavapor coupled to a vacuum pump D-91126, and chiller a MX07R-20HD2E. After solvent elimination, the crude ethanolic extract (EE) that was obtained (13% yield) was then stored at -18°C.

Drugs and pharmacological procedures

Lemon balm EE (100 mg/kg) was dissolved in a solution containing 150 µL of Tween 80, 150 µL of ethanol and 150 µL of Dimethylsulfoxide (DMSO). All these substances (except EE) were obtained from Sigma Aldrich, Brazil. Each resulting preparation was then suspended in 0.9% physiological saline. An equivalent

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preparation of Tween/alcohol/DMSO/saline (this mixture was used to avoid fluid overload, swelling, acidosis and high blood sodium, and to produce the best dilution of the drugs. Saline was used to dilute other medications to be given by injection) was used as vehicle control. These solutions were prepared 24 h prior to being administered via oral gavage, in a volume of 1 mL/kg, having been stored at 4°C. Diazepam (1 mg/kg; DZP; Roche, Brazil) and fluoxetine (10 mg/kg; FXT; Bluepharma, Brazil) were suspended in distilled water containing 2% Tween 80 and physiological saline (DMSO is not necessary in this solution, only for lemon balm EE due to the product consistency), respectively. Both drugs were prepared on the same day as being administered by gavage, in a volume of 1 mL/kg. Lemon balm EE dose selection was based on previous study published elsewhere (Kennedy et al., 2002). The doses of the other substances varied according to the pharmaceutical efficacy of each of them, in accordance with clinical practice.

Caecal ligation and perforation surgery

Rats were weighed and anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg), given intraperitoneally. A 2-cm midline laparotomy was performed to allow exposure of the caecum to the adjoining intestine. The caecum was tightly ligated 5 times with a 4.0-silk suture (Bioline, Brazil) at its base, below the ileocaecal valve, and it was perforated five times through-and-through with a 14-gauge needle (BD, Brazil). The caecum was then gently squeezed to extrude a small amount of faeces from the perforation site, returned to the peritoneal cavity, and the laparotomy was closed with 4.0 silk sutures. The sham-operated rats were submitted to all surgical procedures previously described but the caecum was not perforated. To reproduce the protocol used in surgery procedures on humans, all animals received saline immediately after surgery procedures (50 mL/kg subcutaneous). A mixture of ceftriaxone (30 mg/kg) + clindamycin (25 mg/kg) was administered by intraperitoneal route (i.p.) every 12 h for 3 days. They were kept in groups of 5 animals per cage with free access to food and water, according to the procedure performed elsewhere (Leite et al., 2013).

Treatment protocols

The animals were classified into 2 groups: the sham-operated group and the sepsis group. Each one was divided into 3 subgroups to receive by oral route the control solution (saline); diazepam (1 mg/kg) or fluoxetine (10 mg/kg), as positive controls; or lemon balm EE (100 mg/kg). Animals received daily treatments for 7 days and they were tested one hour after the seventh administration. On the test day, one hour after treatment, the animals were subjected to the behavioural tests. The doses chosen in these protocols were based on the literature or previous experiments from the laboratory.

Experimental procedures

Open field test

To evaluate locomotor activity, animals were tested in an open field (OF), consisting of a wooden square box (60 x 60 x 35 cm) divided into nine equal squares (20 x 20 cm). Each animal was placed in the centre of the field and left to explore freely for 5 min. The number of quadrants crossed with all four paws was used as a measure of locomotion. After each trial, the apparatus was cleaned with ethanolic solution (10% v/v).

Elevated plus-maze test

Measures of anxiety were obtained in the elevated plus-maze (EPM) test. This apparatus is made of wood, comprised of two opposing open arms (50 x 10 cm) and two opposing closed arms (50 x 10 x 40 cm), interconnected by a common central platform (10 x 10 cm). The entire apparatus was elevated 50 cm above floor level, but to prevent falls, both open arms had a 1 cm high plexiglass edge. The animal was placed on the central platform, facing an enclosed arm, and allowed to explore the maze freely for 5 min. The following parameters were scored: number of open and enclosed arm entries, time spent in the central platform, open and enclosed arms. These were used to calculate the percentages of open arm entries (%OAE) and open arm time (%OAT). Arm entry was defined as all four paws in a specific arm (Pellow et al., 1985). After each trial, the EPM was cleaned with ethanolic solution (10% v/v).

Forced swimming test

The forced swimming (FS) test was used, modified from that described by Porsolt et al. (1977). The animal was placed, for 5 min, in a glass cylinder (30 cm in diameter and 50 cm height) containing 40 cm of water at 23±1°C, which forced the rat to swim or float as its hind limbs were unable to touch the bottom. Time spent immobile was recorded only during the last 3 min. Immobility was scored whenever the animal stopped swimming and floated, making only the small limb movements necessary to keep its head above water level. After 5 min, the animal was removed from the apparatus and dried.

Step-down inhibitory avoidance test

The inhibitory avoidance apparatus (Insight, Brazil) is to evaluate aversive memory. It consisted of an acrylic box (50 × 25 × 25 cm), whose floor was made of parallel stainless-steel bars (1 mm diameter) spaced 1 cm apart. A platform (7 cm wide × 2.5 cm high) was placed on the floor against the left wall. The animals were placed on the platform and their latency in stepping down on the grid with all four paws was measured with an automatic device. The animals were submitted to the inhibitory avoidance task using a protocol similar to that described previously (Lucena et al., 2013). During training sessions, immediately after stepping down on the grid, the animals received a 0.4-mA, 1.0-s (controlled by a monitor attached to the equipment) scrambled foot shock. During test sessions, no foot shock was administered and the step-down latency (maximum 180 s) was used as the measure of retention. The animals were submitted to a single training session.

In order to evaluate memory, test sessions were performed 1.5 h after training. The administration of the extract or control solution was performed by oral route 1 h before the training in the inhibitory avoidance apparatus.

Statistical analysis

Data were expressed as means ± mean standard error (s.e.m.) of 10 animals and were analysed using one-way analysis of variance (ANOVA) followed by Tukey's test. Data on the inhibitory avoidance test are shown as median (interquartile range) of step-down latencies. Comparisons of test session step-down latencies

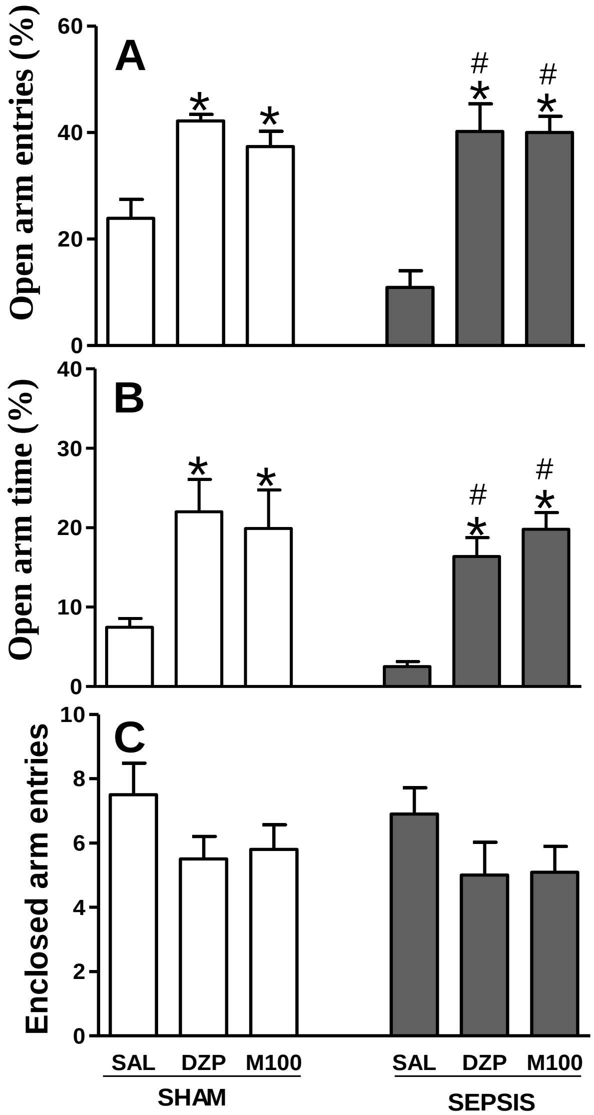

Figure 1. Effects of oral subchronic administration of saline (SAL), diazepam (DZP) and *Melissa officinalis* ethanolic extract (100 mg/kg) on the percentage of open arm entries (A), on the percentage of open time spent (B) and on the number of enclosed arm entries (C) in the elevated plus-maze test on sham-operated and sepsis-surviving rats. Each bar represents the mean ± S.E.M. of 10 animals per group. *$p \leq 0.05$ as compared to the saline treatment of the SHAM group; #$p \leq 0.05$ as compared to the SEPSIS group (ANOVA, Tukey's test).

between groups were performed with the Kruskal–Wallis test followed by the Mann-Whitney test. Data were analysed by Graph Pad Instat® (GraphPad software, San Diego, CA). P-values lower than 0.05 ($P<0.05$) were considered statistically significant.

RESULTS

In the EPM, sham-operated rats that had been exposed to lemon balm EE showed enhanced percentage of open arm entries (Figure 1A): [$F_{(5,59)}$ = 5.00, p<0.05] and percentage of open arm time (Figure 1B): [$F_{(5.59)}$ = 4.20, p<0.05], similar to the animals treated with DZP: % open arm entries (Figure 1A): [$F_{(5.59)}$ =5.06, p<0.05] and % open arm time (Figure 1B): [$F_{(5,59)}$ = 4.91, p<0.05]. In the sepsis-surviving rats, the extract also enhanced the percentage of open arm entries (Figure 1A): [$F_{(5.59)}$ = 5.91, p<0.05] and the percentage of open arm time (Figure 1B): [$F_{(5.59)}$ = 5.07, p<0.05], similar to the animals treated with DZP: % open arm entries (Figure 1A): [$F_{(5.59)}$ =5.96, p<0.05] and % open arm time (Figure 1B): [$F_{(5.59)}$ = 5.00, p<0.05]. The frequency of enclosed arm entries (Figure 2C) in the EPM as well as the number of quadrants crossed in the open field test (Figure 2) were not affected by any treatment and remained constant between groups.

As shown in Figure 3B, FXT treatment was able to reduce the immobility time in sham-operated ([$F_{(5.59)}$ = 3.96, p<0.05]) and sepsis-surviving rats ([$F_{(5.59)}$ = 3.21, p<0.05]) as compared to the group that received the control solution in each group. Lemon balm EE in sepsis-surviving rats modified the immobility time only in the sepsis groups ([$F_{(5.59)}$ = 5.37, p<0.05]).

As shown in Figure 4, the Kruskal-Wallis test followed by the Mann-Whitney test revealed that the lemon balm EE administration was able to improve the memory-facilitating effect produced by sepsis-surviving rats in the model of the step-down inhibitory avoidance test when measuring short- ([$H(4, N=31)$ = 27.10, p<0.0001]) and long-term memory -([$H(4, N=31)$ = 24.80, p<0.0001]).

DISCUSSION

Lemon balm EE was effective in reducing anxiety, depression and cognitive impairment levels produced by sepsis in rodents, showing a property independent of any sedative effect as demonstrated by no interference in locomotion in the open field test and frequency of enclosed arm entries in the EPM, as demonstrated by Figures 1 to 4. Most of the time, the phytomedicine showed a similar effect with the positive controls DZP and FXT, suggesting that the interference in GABA and 5-HT receptors by this extract may be a new alternative for treating neurological dysfunctions produced by sepsis.

Anxiety and depression are the most prevalent mental disorders arising among child, adolescent and adult patients. Both psychiatric illnesses demonstrate marked overlap and co-occurrence (Freeman et al., 2002). Despite this, the prevalence of anxiety is 15 to 25% and the prevalence of depression appears to be higher than 50% (Brown et al., 2001; Lewinsohn et al., 1997). These two disorders may share the same or different vulnerability factors even though anxiety disorders may represent causal risk factors for the development of depression (Goes, 2015; Starr et al., 2014).

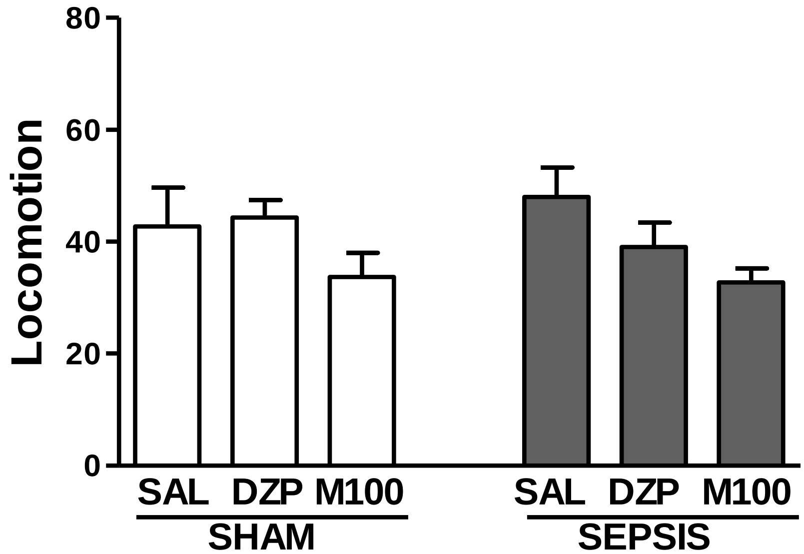

Figure 2. Effects of oral subchronic administration of saline (SAL), diazepam (DZP) and *M. officinalis* ethanolic extract (100 mg/kg) on the number of quadrants crossed in open field test in sham-operated and sepsis-surviving rats. Each bar represents the mean ± S.E.M. of 10 animals per group.

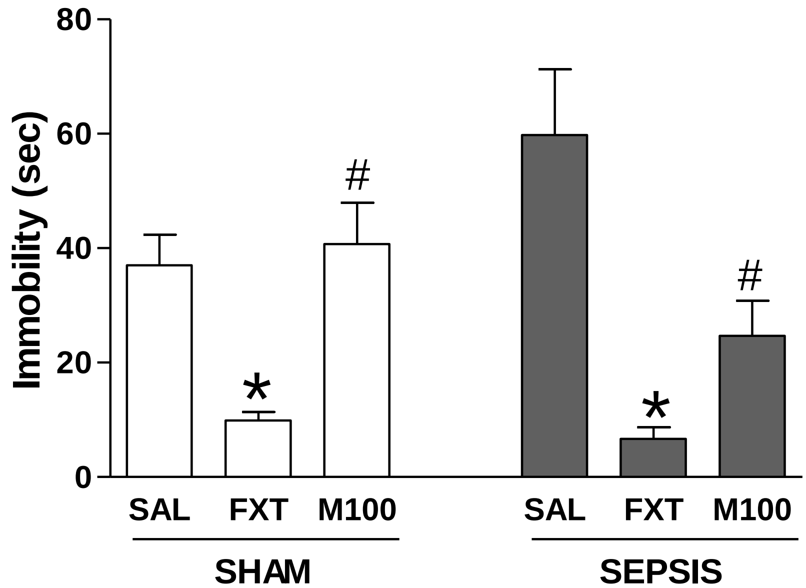

Figure 3. Effects of oral subchronic administration of saline (SAL), fluoxetine (FXT) and *Melissa officinalis* ethanolic extract (100 mg/kg) on the immobile time (in seconds) evaluated in the forced swimming test in sham-operated and sepsis-surviving rats. Each bar represents the mean ± S.E.M. of 10 animals per group. *$p \leq 0.05$ as compared to the saline treatment of the SHAM group; #$p \leq 0.05$ as compared to the FXT group (ANOVA, Tukey's test).

In the context of actual research, it was noted that the sepsis-surviving rats showed suggestive anxiety behaviour that was observed by reduction in the percentage of open arm entries and the time spent in those arms in the EPM, characterizing anxiogenic response. Those rats receiving lemon balm EE maintained a similar response to the

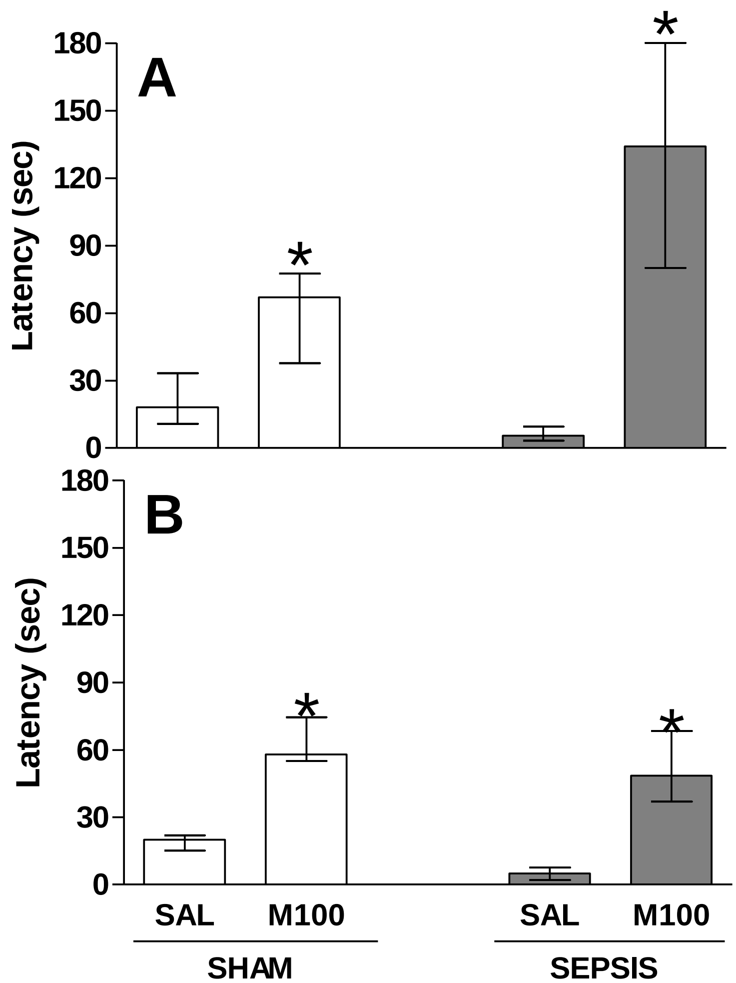

Figure 4. Effects of oral subchronic administration of saline (SAL) and *Melissa officinalis* ethanolic extract (100 mg/kg) on short- (A) and long-term memory (B) in the step-down inhibitory avoidance test in sham-operated and sepsis-surviving rats. Data are shown as the median (interquartile ranges) of 10 animals. *$p<0.05$ as compared to the saline treatment of the respective group (Kruskall Wallis - Mann Whitney).

sham animals. As regards the assessment of depression, the animals showed increased immobility time in the forced swimming test, which was reduced by FXT and the lemon balm EE. These anxiolytic and antidepressant results were not due to either hypoactivity or hyperactivity displayed by animals, since their locomotion was not changed in the open field test and the entries in the enclosed arms in the EPM.

The observed anxiolytic effect of this natural product is congruent with data obtained elsewhere (Kennedy et al., 2006). The results are supported by the fact that rodents normally exhibit a preference for the enclosed arms and avoidance of the open arms of the EPM. Anxiolytic drugs, such as DZP, modulate EPM behaviour in rodents, causing an increase in the percentage of entries and time spent in the open arms of the maze. Therefore, these measures can serve as an index of anxiety (Horii and Kawaguchi, 2015).

Thus, our results are in accordance with previous data, showing anxiogenic and depressive responses in sepsis-surviving rats. Leite et al. (2013) observed that repeated nicotine administration does not alter the survival rate in rats subjected to caecal ligation and puncture and they provide new evidence that nicotine can improve long-lasting memory impairment and anxiogenic-like responses in sepsis-surviving animals. Tuon et al. (2007), in turn, demonstrated in sepsis-surviving rats, a significant increase in the immobility time as compared to the sham-operated rats. The imipramine was able to reduce this evaluated parameter, with no locomotion impairment. In the experiments, the FXT, a selective serotonin reuptake inhibitor, showed similar results.

Lemon balm is known for its mild sedative properties (Anheyer et al., 2017), even following a single administration (Soulimani et al., 1991). In our study, such behaviour was not observed. The fact that the lemon balm EE and DZP did not generally alter quadrant crossings and enclosed arm entry in the sham and EPM tests, respectively, suggests that the psychoactive effects observed in the present study may not be due to changes in the animals' locomotor patterns.

Regarding the cognitive aspect, animals that survived the sepsis showed a loss of short- and long-term memory, which was characterized by the decrease in length of time spent in the inhibitory avoidance platform. The administration of lemon balm EE made the animals spend more time on the platform, characteristic behaviour of memory retention.

Several studies have recently shown the importance of phytotherapies for treating behavioural dysfunctions and cognitive problems in humans (Anheyer et al., 2017; Kennedy et al., 2002; Ross, 2015), even though there is little information about the possible pharmacodynamics involved in the pharmacological effects. For *M. officinalis*, it is well documented that its effects could be related to the active components that have been identified as monoterpenoid aldehydes, monoterpene glycosides, flavonoids and polyphenolic constituents (Shakeri et al., 2016; Wightman, 2017).

Substantial progress has been made in our understanding of how some herbal medicines can ameliorate behavioural and cognitive processes in patients with different neurological impairments. As previously mentioned, nature has sources for various types of treatment, providing effective antimicrobials to treat infections caused by bacteria, viruses and fungi, while offering therapeutic support to multiple organs and body systems. On account of this, in this present research, we also do not rule out the possibility that lemons balm EE have interfered in aspects directly related to inflammation, since it possesses potential anti-inflammatory activities, supporting the traditional application of this plant in treating various diseases associated with inflammation and pain in more peripheral areas (Bounihi et al., 2013) and brain areas, whose active principles can probably be carried through the cerebral blood flow and interact with several neurotransmitters and signalling pathways involving kinase enzymes (Wightman, 2017).

Conclusion

Although, there is a wide variety of a drug to treat microbial infections, it is important to consider the frequency and severity of adverse effects. Moreover, as the arsenal of available antibiotics in the pharmaceutical market is increasingly being depleted due to microbial resistance, there is a need for more alternative and less toxic treatments. Thus, considering the results obtained here, it is possible to suggest that the extract from lemon balm can be considered a potential alternative for the treatment of diseases, which have similar (or no similar) reliability as conventional drugs DZP and FLX.

CONFLICTS OF INTERESTS

The authors have not declared any conflict of interests.

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Medicinal plants and herbalist preferences around Bwindi Impenetrable National Park

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Many people in Uganda, particularly in the rural areas depend on herbal medicine for health care. Although, medicinal plants are collected from different habitats, most of them are collected from the wild. Claims indicate that men use more forest products than women; and that women prefer younger vegetation succession stages as compared to those preferred by men for harvesting medicinal plants. However, there was no documented evidence to ascertain these claims. This study documented medicinal plants and investigated the influence of gender in the collection of these plants around Bwindi Impenetrable National Park. The plants used by various categories of herbalists were collected, identified and listed. The differences between herbalist categories regarding medicinal plant collection and use were studied using both qualitative and quantitative participatory rural appraisal (PRA) methods. Two hundred and ninety five species encompassing all growth forms were listed with herbs and shrubs being most frequently used and majority (70%) of these occurred outside the forest. Most of the mentioned plants were reported to be used in mixtures while leaves were reported as the most frequently used plant part.

Key words: Medicinal plants, Bwindi Impenetrable National Park, conservation.

INTRODUCTION

The art of traditional medicine has been practised for many years in the third world countries including Uganda (Malini et al., 2013; Ondicho et al., 2015). In Africa, communities have relied upon the spiritual and practical skills and knowledge of the traditional medical practitioners for many years (Kiringe, 2006b; Winkler et al., 2010). Pharmacognosy has since then evolved into a sophisticated science that is emerging to the forefront

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of drug discovery. This knowledge has been retained in some cultures such as, the Asian culture because the information was handed down in written form unlike in Africa and South America where knowledge was passed on by word of mouth. To date, knowledge is still getting lost due to death of the elderly people who are custodians of indigenous knowledge, migrations, regional conflicts and urbanisation among other causes. Traditional medicine is more accessible than modern health facilities to most of the population in the developing world such as Uganda because it is relatively inexpensive, locally available, usually accepted by the local communities and poor healthcare delivery systems (Sambo, 2010; Osemene et al., 2011).

In Uganda, people became more aware of the importance of traditional healing systems. Medicinal plants were reportedly widely used by traditional midwives, bonesetters, spiritualists and other categories of Traditional Health Practitioners (Tabuti, 2008). There was also a marked shift from medicinal plant collection by herbalists towards commercial harvesters including international pharmaceutical companies; and this was partly due to the high potential economic value of drugs derived from medicinal plants globally (Lev, 2006a; Omonona et al., 2012). It was observed that 74% of the plant-derived compounds currently used in pharmaceuticals retained the same or similar use by traditional healers (Moran, 1997). Development of western drugs from medicinal plants, therefore, depends on cultural as well as species survival (King, 1996).

Herbalists around Bwindi Impenetrable National Park tend to believe that wild harvested medicinal plants are more effective than cultivated ones (Bekele, 2007; Cunningham, 2001; Ngarivhume et al., 2015). Alongside the global concern about the increasing need for healthcare (Bodeker, 1994; Delloite, 2015), there is need to ensure the sustainable use of wild medicinal plants, their safety and efficacy. Reports by UNESCO show that the wild harvest supply of traditional medicine is failing to meet the ever-increasing demands of the people (WHO, 2005; Bandaranayake, 2006; FAO, 2016).

A change in land use to cater for agriculture, agroforestry and fuel supply, whilst increasing weedy species used medicinally, has resulted in a decline in the total area of natural vegetation available as a source of herbal medicines, especially forest species (Cunningham, 1996). Phillips et al. (1994) demonstrated that some vegetation types are much more important to local people than others in terms of use value. However, it has not been demonstrated quantitatively that tropical medicinal plants are concentrated in primary as opposed to disturbed forest vegetation. Voeks (1996) reported that healers demonstrated a strong preference for disturbed over primary forest and that there may be an intrinsic medicinal value of disturbance species. Iwu (1993) reported that Africa has the highest rate of deforestation in the world. Habitat conversion threatens not only plant resources but also traditional and cultural diversity and the associated knowledge of medicinal value of several species. This study was carried out around Bwindi Impenetrable National Park to establish how the use of medicinal plants by traditional birth attendants is compared to that by other herbalists in terms of sustainability and habitat preference and specificity of collection. Information obtained is important in utilisation and conservation of medicinal plants used by communities around Bwindi impenetrable National Park.

MATERIALS AND METHODS

Study area

Bwindi Impenetrable National Park is located in South-Western Uganda at longitude 29° 35′ to 29° 50′ E and latitude 0° 53′ to 1° 8′ S. It is situated at the edge of the Western Rift Valley and occupies the highest block of Rukiga Highlands. It lies at the Ugandan border with the Democratic Republic of Congo, about 29 km north of Kabale town, 18 km north of Kisoro and 40 km South-East of Lake Edward. The park shares boundaries with three districts in south-western Uganda (Kanungu, Kisoro and Kabale). It is bordered by parishes with high population density and agricultural activity which is particularly notable in the districts of Kanungu and Kabale. The communities in these areas are composed primarily of two ethnic groups dominated by Bakiga who are a group of Bantu speaking agriculturalists with a long history of association with the forest and still use many forest products. The other group are the Abayanda who are concentrated in the extreme south-western corner of Uganda within 2 parishes of the park boundary (Kabananukye and Willy, 1996). In this study, information was collected from five parishes around the park namely; Kitojo, Nteko, Mpungu, Nyamabale and Rutugunda.

Participatory rural appraisal (PRA) methods were the main methods employed in obtaining the ethnobotanical information required for this study. Both qualitative and quantitative approaches were used to collect information on medicinal use of the plants. The qualitative methods included informal conversations, semi-structured interviews, free listing, field excursions and visits to local markets while the quantitative methods included preference ranking and pairwise ranking. Local authorities and established TMP and TBA committees were used to identify and locate the locally recognised herbalists in each of the selected parishes. A total of 100 people were interviewed.

Informal conversations involved participating in conversations with local people and directing the conversations towards the issue of medicinal plant use without formally explaining the intention to carry out a study and the information collected would be recorded immediately after the conversations. Meeting places included churches, women-group meetings, homes, drinking places, pathways and gardens. Field excursions involved a walk together with herbalists as they went to collect herbal medicines especially from the forest. This was mainly possible in multiple-use zones where group arrangements were made for collection of medicinal plants from the forest from time to time. The information gathered on such occasions supplemented that from other methods on local plant names, parts used and other aspects of medicinal plant use. In each of the parishes visited during this study, there is at least one market place recognised by the local people and there is a market day at least once a week. This provided an opportunity to carry out market surveys in each of the parishes visited. In each of the markets visited, the local names, growth forms and parts of the medicinal plants being sold as well as the gender of the vendor were recorded. Semi-structured interviews were conducted in the local language (Rukiga) with each of the respondents selected for this study by use of interview schedules. The place and time of the interview were arranged well in advance at the interviewee's convenience to avoid inaccuracy resulting from inconveniencing the respondents. Free listing was carried out to obtain a list of medicinal plants given by each of the 100 people interviewed in the study. For each of the plants listed, the part(s) used and, where possible, the disease(s) treated were recorded. With each interviewee, voucher specimens of the plants listed were collected and identified at Makerere University herbarium and deposited there. Pair wise and preference ranking techniques were used to study the ranking given to each of the different types of habitats. The ranking was done with 80 respondents, 20 from each of the categories of herbalists. Each respondent ranked six different types of habitats in their order of preference. Preference ranking was done in order of preference of medicinal plant collected to obtain total ranks by the total number of rankers. While pair wise ranking was done to obtain a total score computed for each type of habitat. The higher the total score, the more preferred, the given type of habitat.

Ethical approval

Ethical clearance was received from the Faculty Ethical Review Committee of Kyambogo University.

Data analysis

Epi-Info version 6 was used for both data entry and analysis. Frequencies were produced and Chi-square test was used to test for association between herbalist categories and growth forms of plants, habitats and parts used. The rest of the data were summarised and analysed manually.

RESULTS

Majority of the respondents (99%) revealed that they use medicinal plants to treat themselves and/or other people in or outside their families. Only one person said she did not use medicinal plants though she was able to list some plants that she knew were medicinal. Most of the respondents (90%) had used medicinal plants for at least five years. Majority of the respondents (58%) were above 50 years of age. Many of these (51%) were not educated, only 2% had secondary education.

Medicinal plants in and around Bwindi

About 288 medicinal plant species were listed. Of these, 257 were identified belonging to 204 genera in 84 families. Sixty-nine species could not be collected as most of them were reported to be so rare and difficult to get. Thirteen species could not be identified and have, therefore, been represented by their local names given by respondents. The average number of plants mentioned per herbalist was 20, while the highest and least numbers of species mentioned by herbalist were 165 and 2, respectively.

According to Table 1, the most frequently mentioned genera by respondents were *Vernonia* (8 species), *Solanum* (6 species), *Pentas*, *Ficus, Crotalalia* and *Crassocephalum* respectively. *Hibiscus fuscus* was the most frequently mentioned species. The plants were collected in different habitats including; garden, early fallow, mature fallow, bushy thicket and forest. Most (30%) species mentioned by respondents were collected from the mature fallow; followed by the bushy thicket and forest at 21.5 and 21.2%, respectively. Among the respondents, the male non-specialists (MNS) mentioned *Rytiginia* species more often than all the other herbalist categories.

All plant parts were found to be harvested for medicine in different proportions. Leaves were the most frequently harvested (70.9%) plant part, followed by roots (10.8%) as shown in Figure 1.

Growth forms

All plant growth forms of medicinal plants including herb, shrub, tree, liana and grass were mentioned by respondents. One fungus was mentioned among the medicinal plants as seen in Figure 2. Majority (49.1%) of the plant species mentioned by respondents were in form of herb, followed by shrubs (26.1%).

Commercial harvest of medicinal plants

Some medicinal plants sold in the local markets around Bwindi Impenetrable National Park that were visited during the study were listed; and their habitats and parts sold are documented as shown in Table 2.

Differential medicinal plant use by herbalists

Differences in medicinal plant use between the different categories of respondents were observed. The categories included male herbalists (MH), traditional birth attendants (TBA), female herbalists (FH) and male non-specialists (MNS). The differences were noted in the following aspects: average number of plants mentioned per respondent, number of species used singly, plant parts used, habitats from which herbalists collect medicinal plants and growth forms of plants used by respondents. The differences in the numbers mentioned are given in Table 3.

The specialist herbalist categories (TBA and MH) listed more plant species than the non specialist categories (FH and MNS). The male herbalists mentioned more medicinal plants than the rest of the categories. The number of plants used singly by non-specialists is higher than that used singly by other herbalist categories.

Although, the p value does not indicate significant differences between the different categories of herbalist as far as the use of various growth forms of plants is

Table 1. Species scientific name, local name, family, habitat and part used of thirty medicinal plant species most frequently mentioned by the respondents around Bwindi impenetrable National Park.

Plant scientific name, local name, family, habitat	Part used	Number of responses				
		TBA	MH	FH	MNS	Total
Hibiscus fuscus, Omusinga,(Malvaceae) MF	L	14	21	14	08	57
Leucas martinicensis, Akanyamafundo, (Lamiaceae) MF	L	14	16	12	06	48
Myrica salicifolia, Omugyegye, (Myricaceae) F	L,B	10	18	10	08	46
Pentas longiflora, Esigara, (Rubiaceae) MF	L,R	12	19	11	07	46
Leonotis nepetifolia, Ekicumucumu, (Lamiaceae) EF	L	12	14	08	08	43
Oxalis corniculata, Obunyunyambuzi, (Oxalidaceae) EF	W	10	15	10	03	38
Lantana trifolia, Omuhukye, (Verbanaceae) BT	L, Fl	08	16	08	05	37
Lysimachia ruhumeriana, Akanyabuhengere, (Primulaceae) EF	W	12	12	07	05	36
Impatiens stuhlmannii, Omurembe, (Balsaminacaceae) MF	W	12	16	06	01	35
Galium sparium, Kaboha, (Rubiaceae) MF	W	09	15	08	02	34
Helichrysum schimperi, Ekyeeza, (Asteraceae) MF	L	09	15	07	02	33
Peponium vogelii, Omugoshora, (Curcubitaceae) MF	L	08	13	07	03	31
Dombeya goetzenii, Omukole, (Tilliaceae)F	L,B	08	14	05	02	29
Indigofera erecta, Omusoroza, (Papilionaceae) EF	L,R	07	10	09	02	28
Maesa lanceolata, Omuhanga, (Myrisinaceae) F	W	08	11	04	04	27
Bothlioclines longipes, Ekyoganyanja, (Asteraceae) MF	L	08	09	07	02	26
Rytiginia sp., Nyakibazi, (Rubiaceae) F	W	05	07	02	11	25
Bidens pilosa, Enyabarashana, (Asteraceae) EF	L	07	10	06	02	25
Pennisetum papilium, Ekibingo, (Poaceae)BT	L	07	10	06	02	25
Vernonia auriculifera, Ekigaragara, (Asteraceae)T	L	12	04	04	01	21
Gouania longispicata, Omufurura, (Rhamnaceae)F	L, R	06	07	05	03	21
Vernonia lasiopus, Omujuma,(Asteraceae) MF	L	04	07	05	04	20
Physalis peruviana, Omututu, (Solanaceae) EF	L	07	04	06	02	19
Piper guigeense, Rukookota, (Piperaceae) F	R,B	02	07	03	07	19
Pictostachys eminii, Ekisindookwa, (Lamiaceae)MF	L	04	06	04	04	18
Plectrunthus barbetus, Ekicuuncu. LamiaceaeBT	L	06	05	05	02	18
Albizia gummifera, Omushebeya, (Ceasalpinaceae)F	L	09	07	01	01	18
Cissus adenocaulis, Ekibombo, (Vitaceae)BT	W	05	10	03	00	18
Dichrephalia integrifolia, Omubuza, (Asteraceae)EF	L	06	08	02	01	17
Pavonia scimperiana, Eihoza (Malvaceae) MF	L	04	09	03	01	17

Habitats: MF- Mature fallow; BF- bushy thicket; F- forest, EF- early fallow; G- garden. Plant part: L- leaves; B- bark; R- roots; FL- flowers; W- whole plant. Category of respondents: TBA- traditional birth attendants; MH- male herbalist; FH- female herbalists; MNS-male non-specialist.

Table 2. Scientific name, habit and plant part sold in the local markets around Bwindi Impenetrable National Park.

Plant species	Habit	Plant part sold
Anisopappus africanus	Herb	Leaves
Cissus adenacaulis	Liana	Roots
Hallea rubrostipulata	Tree	Stem bark
Maesa lanceolata	Tree	Stem Bark
Myrica salicifolia	Tree	Stem bark
Ocotea usambalensis	Tree	Stem bark
Piper guineense	Liana	Root
Symphonia globurifera	Tree	Stem bark

Most of the plants involved in the commercial harvest were trees. In all the plants except *Anisopappus africanus*, *Cissus adenacaulis* and *Piper guineense,* the stem bark was sold.

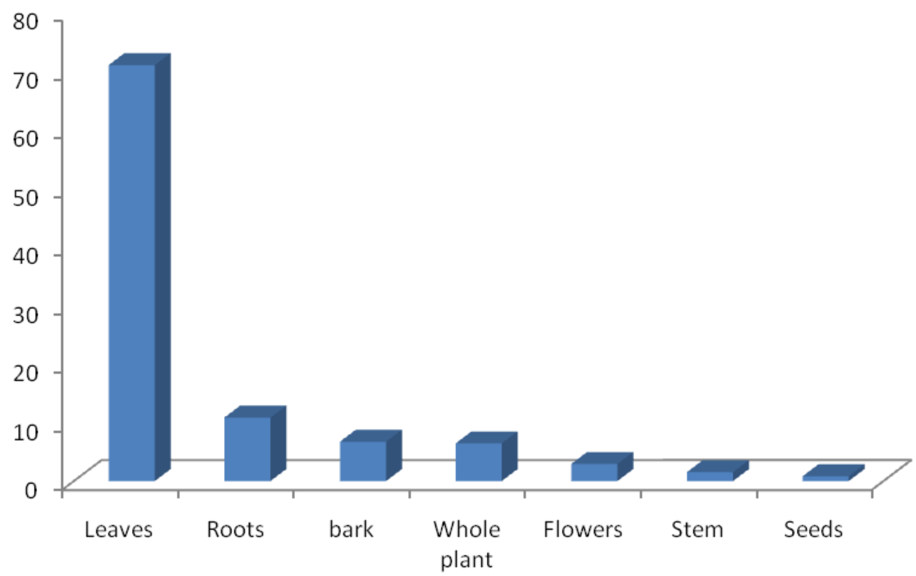

Figure 1. Preference of the different medicinal plant parts used by the respondents around Bwindi Impenatrable National Park.

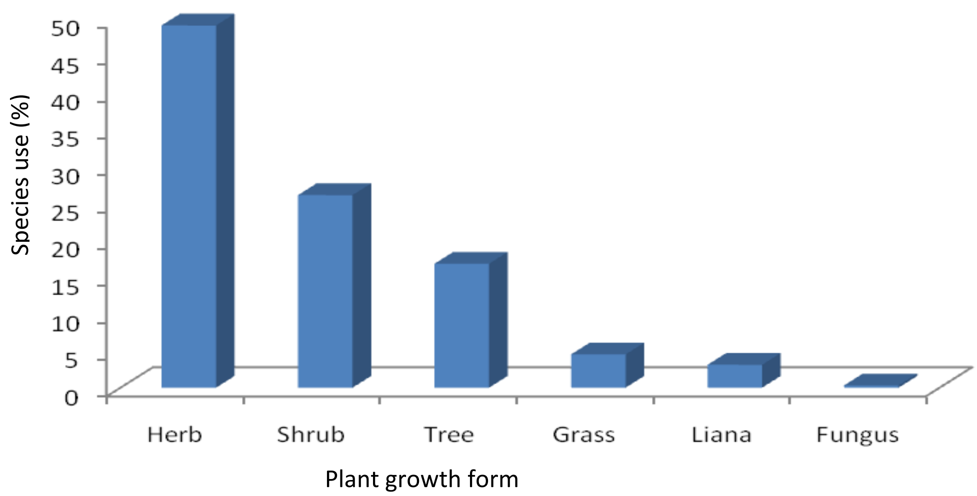

Figure 2. Percentage of species use with the various medicinal plant growth forms mentioned by the respondents around Bwindi Impenetrable National Park. N=288.

concerned, the herb is the most used growth form for all herbalist categories. It can also be seen from Table 4 that the specialist herbalist categories (TBA and MH) collect more proportions of herbaceous plants than the non specialist categories (FH and MNS) that, comparatively, collect more proportions of trees than the former.

Differences in use of plant parts

The p value < 0.05 indicates highly significant differences between various herbalist categories. Although, leaves are the most frequently used plant part in all the herbalist categories, they are most frequently used by traditional birth attendants (TBA) and women herbalists (FH) than their male counterparts. It can also be seen that the male herbalist categories (MNS and MH) collect more from the root and bark parts of the plant as compared to their female counterparts.

Differential habitat preference by herbalists

The proportions of plants collected by the different

Table 3. Plants used singly in treatments and frequency of mention of medicinal plants used by the different categories of respondents around Bwindi Impenetrable National Park.

Herbalist category	Average no. of plants mentioned	Total no. of plants used singly	A highest number mentioned	Least number of plants mentioned
TBA	20	29	39	3
FH	18	26	65	5
MH	34	33	162	3
MNS	9	22	18	2

Category of respondents: TBA- traditional Birth attendants; MH- male herbalist; FH- female herbalist; MNS- male non-specialist.

Table 4. Percentage of plant growth forms mentioned by different categories of herbalist respondents around Bwindi Impenetrable National Park.

Herbalist category	Percentage of plants mentioned by growth forms						
	Shrub	Tree	Herb	Liana	Grass	Fungi	Total number
TBA	23.6	15.7	53.4	2.8	4.5	0.0	184
WH	22.2	25.9	47.6	1.9	2.4	0.0	179
MH	22.8	16.7	53.3	3.3	4.1	0.3	258
MNS	25.5	24.5	44.7	2.1	3.2	0.0	93

Category of respondents: TBA- traditional birth attendants; MH- male herbalist; FH- female herbalist; MNS- male non-specialist P=0.374 (15 d. f.)

Table 5. Percentage of medicinal plant/ part used by different herbalist categories around Bwindi Impenetrable National Park.

Herbalist category	Percentage of plants used by plant parts							
	Leaves	Whole plant	Bark	Roots	Flowers	Seeds	Stem	Total number
TBA	85.1	7.8	4.4	2.2	0.4	0.2	0.0	184
FH	84.1	7.1	5.7	2.9	0.0	0.2	0.0	179
MH	78.9	9.6	6.3	3.7	0.3	0.4	0.7	258
MNS	70.3	7.2	16.3	6.2	0.0	0.0	0.0	93

P=000 (18 d. f.); Category of respondents: TBA- traditional birth attendants; MH- male herbalist; FH- female herbalist; MNS- male non- specialist. Habitats: MF- mature fallow; F- bushy thicket; F- forest, EF- early fallow; G- garden

herbalists from the various habitats are presented in the Table 5. The value of $p<0.05$ from the Chi-square comparison results in Table 6 indicate significant differences between the various herbalist categories regarding their use of the various habitats for collection of medicinal plants. The highest percentage of plants used by the male herbalists and non-specialists are commonly collected from the forest. On the other hand, most plants used by TBA and women herbalists are collected from mature fallow and bushy thicket, respectively. The garden is the least source of medicinal plants for all the herbalist categories. Of the four herbalist categories, the male non specialists mentioned the least number of plant species from gardens followed by the male specialists. While plants mentioned by the female herbalist categories (TBA and FH) were evenly distributed among various habitat categories, more than 50% of those mentioned by their male counterparts (MH and MNS) were collected from the bushy thicket and forest vegetation habitats. The MNS mentioned the highest percentage of plants from the forest.

Habitat categories

Positions of the various habitat categories (stages of succession) as obtained from preference and pairwise ranking by the respondents are given in Table 7.

Similarity was observed in the habitat preferences of traditional birth attendants and the women herbalists ranking mature fallow and bushy thicket, respectively, in the first position. On the contrary, both male herbalists

Table 6. Percentage of plants collected from the various habitants mentioned by the different herbalist categories around Bwindi Impenetrable National Park.

Herbalist category	Percentage of plants from each habitant type					
	MF	EF	BT	F	G	N
TBA	24.8	22.7	21.3	21.7	9.5	184
WH	24.1	23.2	24.1	20.0	8.6	179
MH	21.1	18.0	22.8	28.7	9.4	258
MNS	15.2	18.1	25.0	37.8	3.9	93

P=0.002 (15 d.f.); Category of respondents: TBA- traditional Birth attendants; MH- male herbalist; FH- female herbalist; MNS- male non- specialist. Habitats: MF- mature fallow; BF- bushy thicket; F- forest, EF-early fallow; G- garden.

Table 7. Average positions of the habitats as ranked by different herbalist categories around Bwindi Impenetrable National Park.

Herbalist category	Habitats					
	GP	G	EF	MF	BT	F
TBA	6th	4th	3rd	1st	2nd	5th
FH	6th	5th	3rd	2nd	1st	4th
MH	6th	5th	4th	3rd	2nd	1st
MNS	6th	5th	4th	3rd	2nd	1st

Category of respondents: TBA- traditional birth attendants; MH- male herbalist; FH- female herbalist; MNS- male non-specialist. Habitats: GP ground prepared for planting; MF- mature fallow; BT- bushy thicket; F-forest, EF- early fallow; G- garden

and the male non-specialists ranked the forest highest, while habitats ground prepared for planting and garden were ranked last and second last, respectively, by all the herbalist categories. Results from pair wise ranking further reveal that these scored more highly with TBA than with the rest of the herbalist categories.

DISCUSSION

Ethnobotany research that explicitly tests hypotheses facilitates its conceptual development. However, most documented studies simply on total uses reported by respondents; assign importance values by subjective *a posteriori* processes; or simply calculate the percentage of useful plants. Yet quantitative ethnobotanical surveys can be powerful diagnostic tools for conservationists (Phillips et al., 1994). This study used both qualitative and quantitative approaches to conceptualise the output.

Many communities worldwide, Uganda inclusive continue to use herbal medicine in health care delivery. This is because communities believe that plants are efficacious and safe and hence widely acceptable to the people (WHO, 2005). Cunningham (1996) found that TBAs around Bwindi Impenetrable National Park used 154 plant species. According to him, TBAs around BINP use medicinal plants for symbolic purposes; preventing premature labour, facilitating labour, removal of placenta, treating swollen breasts or improving lactation and treatment of internal parasites among other treatments. In this study, more plant species (184) used by TBAs were documented. Reasons for the higher number of plants used could be the increased population, as well as the enlarged actual needs of the people. The individual medicinal plant lists in this study indicate a high level of medicinal plant use and knowledge around BINP as indicated by the high average number of medicinal plant species mentioned per respondent. An average number of plants mentioned by the male non-specialist herbalists and the fact that none of the respondents in the study failed to give any medicinal plant names, may indicate that every person in the study area has at least some knowledge on medicinal plants. Some of the people contacted during this study testified that the people around BINP use medicinal plants to treat many ailments since the health centres are located very far from most communities in the area. Only one respondent in this study revealed that she did not use medicinal plants, her husband was a medical assistant and always provided western medicine whenever a family member was sick. Still other people revealed that there are certain illnesses which cannot be cured in hospitals but can be cured using traditional medicine.

The study indicated that most of the medicinal plants mentioned were used in mixtures. This means that respondents collect portions from several plant species in order to make a mixture which is given to the patient. It is believed that, in this case, less amounts of plant material are collected from the individual plant species as compared to the case where a particular plant is used singly to treat an ailment. This indicates, in part, that the amount collected from each plant species may be small enough to allow for the sustainable use of the medicinal plant species. It is also evident from the study that the most frequently used growth form of plants is herbs.

Most of the medicinal plant species listed in the study were found to grow mainly in the mature fallow. The reason for this could be accessibility of the habitat as well as the tendency for herbalists to believe that the older the habitat, the stronger the medicine collected from such a habitat. On the other hand, the forest and bushy thicket did not contribute as much as the mature fallow probably because these habitats are commonly far away from homes. People in Kigezi are agriculturalists and tend to cultivate the areas around their homes. This makes it difficult to find vegetation older than what is termed mature fallow in this study. It has been reported that clearing of forests in the Rukiga highlands is thought to have coincided with the influx of Bantu-speaking agriculturalists about 2,200 years ago (Taylor, 1990). According to some of the people contacted in this study, it is traditionally believed that the older the plant, the more important is the medicine prepared from it. This view was expressed by both male and female herbalists. Consequently, herbalists tend to collect medicinal plants from the oldest type of vegetation nearest to their reach. The same reasons could have accounted for the least number of medicinal plants from gardens which also agree with earlier findings by Fuller (1991) and Omonona et al. (2012) that herbalists tend to believe that wild harvested plants are more effective than cultivated plants in herbal medicine. These results are similar to earlier findings by, for instance, Voeks (1996), who observed that secondary forest, was the preferred habitat for local healers and that secondary forests medicinal plants were prescribed for a greater number of ailments.

In earlier studies, it was found that leaves were the most used plant part by herbalists' around BINP (Cunningham, 1996). This study revealed that leaves are the most frequently used plant part by all the herbalist categories, although the females use more leaves as compared to their male counterparts who use more stem bark and roots. There is a possibility, therefore, that medicinal plant collection around BINP is still dominated by collection of leaf material which is less likely to endanger the growth of most of the medicinal plants involved. In Africa, in general and Uganda in particular, women are traditionally considered to be weaker than the men in terms of energy. Consequently, men tend to perform more energy-requiring tasks than women. In reality, harvesting the bark and roots requires more energy than harvesting leaves from plants, especially trees. Biologically, the collection of roots and stem bark from plants endangers the plant life more than collection of leaves. This means that, in this context, medicinal plant use by female herbalists may be more sustainable than that by their male counterparts.

The study showed that the highest percentage of plants used by male herbalists are collected from the forest vegetation while that by the female herbalist are collected from the mature allow and bushy thicket. One of the reasons for these differences maybe because women are always busy at home and do not have enough time to move to the forest to collect medicinal plants. It has been observed that in addition to being providers and promoters of health through their reproductive and productive roles, and related activities, women are actually over burdened in terms of workload and long periods of work (Nakyanzi, 1997; Bezner et al., 2016). It could also be due to the fact that women are always in contact with agricultural and land and can, therefore easily identify medicinal plants in these habitats. The male respondents tend to spend much of their time with forest vegetation while they hunt wild animals, look for timber, and open up ground for agriculture and other activities. This makes them know more about forest vegetation and consequently they collect most of their medicines from this vegetation. The forest in this area is the national park where only registered traditional medical practitioners are allowed to harvest medicinal plant material. It appears that people who are not allowed to get plant material from the national park actually harvest bigger proportions of their medicinal plants from there as compared to those that are allowed to do so, that is, the TBA and MH. However, the actual numbers show that MNS use the least number of plant species.

Looking at the harvest of different growth forms of plants by various herbalist categories and in the light that herbs produce more individuals than trees at a given time, it appears that the plant harvest by non-specialist herbalist categories (FH and MNS) may be more dangerous to the plant species than that by the specialist categories (MH and TBA). This is because the former collect higher proportions of trees than the latter who, in turn, collect higher proportions of herbs. The fact that MNS registered the least number of medicinal plants used may mean a big burden of harvesting on these few plants. Moreover, almost all plants used by the MNS are also used by other categories as well. In this study, *Tephrosia vogeli* and *Raphiostylis beniensis* were the only species mentioned exclusively by the MNS. Since the MNS were not allowed to harvest from the park under the multiple-use system, it means that the plant material they harvested was not recorded in the system. This could mean that the amount of *Rytiginia* species material actually harvested exceeded that expected to be harvested per year in the multiple use zones. This has also been found to exceed the amount allowed per year by far (Kamatenesi, 1997). The fact that female herbalists use more herbaceous plants than their male counterparts may be another reason to believe that medicinal plant use by female herbalist maybe more sustainable than that by their male counter parts.

Results from pair wise and preference ranking also indicate that there are differences in habitant preferences between male and female herbalists. By observation that habitat category(GP) ground prepared for planting scored higher in female herbalists than in their male counterparts, is likely to stem from the fact that these habitats are more important in gardens which occupies the women most of the time. The male herbalists ranked the forest vegetation first, while this was ranked second last and third last by FH and TBAs, respectively. The reasons given for preferring the forest by some of the men were that the forest harbours the strongest and most important medicines and that there is enough secrecy in this environment. The women on the other hand indicated that they go to the forest to harvest those medicinal plants that cannot be obtained outside the forest. The study indicates that the female medicinal plant habitat preference tends to decrease with stages of vegetation succession, while that of their male counter parts tends to increase as the vegetation grows older. The kinds of ailments and diseases treated by men and women also tend to vary bringing about some of the differences observed in habitant preferences. From observation, men try to handle more complicated cases which may have failed in allopathic medicine but do not necessarily require emergency. Women, on the other hand, mainly attend to emergency cases such as births and child sicknesses that do not allow time for someone to move a long distance in order to collect medicinal plants. Consequently women have learnt about many medicinal plants in their surroundings which mainly consist of younger stages of succession in vegetation.

Conclusion

Medicinal plants are an important source of health care for almost all the people around Bwindi Impenetrable National Park. Most of the medicinal plants are not planted in gardens but harvested from fallows, thickets and forests. The study also found out that some habitats are more important to herbalists than others as far as collection of medicinal plants is concerned. There is gender distinction in habitat preference for medicinal plant collection by herbalists. Gender also influences parts of medicinal plants used by herbalists in Bwindi area. It was observed that male herbalists often use more destructive plant collection methods than their female counterparts.

Conflict of interests

The authors declare that no conflict of interest exists.

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